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Nutritional flexibility of oligotrophic and copiotrophic Antarctic bacteria with respect to organic substrates

A.C. Upton and D.B. Nedwell

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1. SUMMARY

The nutritional flexibility of oligotrophic and copiotrophic bacteria from an Antarctic freshwater lake sediment was investigated. Bacteria isolated on plates of oligotrophic and copiotrophic media were replica plated onto media containing different substrates, and their ability to utilise the different substrates was determined. The oligotrophs were shown to be able to utilise a significantly broader range of organic substrates than the copiotrophs, consistent with the idea that nutritional flexibility is adaptive for oligotrophic bacteria.

2. INTRODUCTION

Two principal definitions of oligotrophic bacteria have been proposed. Kuznetsov et al. [1] tentatively classified bacteria isolated from oligotrophic habitats as those that developed at the first cultivation on media with minimal organic matter of about 1–15 mg $C.l^{-1}$ and that grow on such media at subsequent recultivations although they can grow on richer media. Poindexter [2] found it more appropriate to define a habitat in terms of the average flux of energy through the environment, and conceived oligotrophic bacteria as those which can multiply in habitats of low energy flux of a fraction of a mg $C.l^{-1} \cdot d^{-1}$. In copiotrophic environments, the nutrient flux is at least fifty times greater and does not drop to zero for prolonged periods.

These concepts may be summarised as a 'fast and famine' oligotrophic existence [2] and a copiotrophic 'feast and famine' existence [3]. In reality bacteria in the two groups are probably not separated into distinct groups [4,5] but represent extremities of a continuous scale covering the complete range of physiological ability.

The growth of oligotrophic bacteria is primarily limited by the availability of assimilable organic substances. Poindexter [2] proposed that bacteria adapted to oligotrophic conditions might be expected to possess uptake systems for a more diverse variety of substrates than bacteria adapted to copiotrophic conditions, so maximising simultaneous utilisation of a multiplicity of substrates. The characteristics of oligotrophic bacteria, and

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mechanisms facilitating survival in oligotrophic environments, have been extensively reviewed [6–9]. This present work investigated whether bacteria isolated on an oligotrophic medium were able to use a greater variety of organic substrates than those isolated on a copiotrophic medium.

3. MATERIALS AND METHODS

3.1. Isolation of oligotrophic and copiotrophic bacteria

Source of isolates. Freshwater sediment was collected in February 1985 in a sterile 250 ml polypropylene bottle from Heywood Lake, Signy Island (60°40′S, 45°35′W), a member of the South Orkney Islands in the maritime Antarctic. The sample was stored at −20°C prior to use.

Culture of isolates. The freshwater sediment was thawed overnight at 4°C before a ten-fold dilution series was prepared aseptically. The diluent was the Griffiths and Morita [10] replication medium with the carbon source and agar omitted and the volume of the distilled water in the basal medium adjusted to account for any reduction in volume due to the ommissions. Subsamples (0.1 ml) of each dilution were aseptically spread with sterile glass spreaders onto triplicate pre-cooled plates of casein peptone starch (CPS) [11] and oligotrophic enrichment (OEMS) [12] isolation media. The copiotrophic isolation plates were incubated for 10 weeks (the first 4 weeks at 10°C then at 4°C) and the oligotrophic isolation plates for 13 weeks at 10°C until there were no further increases in the number of colonies on the plates.

3.2. Isolation media

The copiotrophic CPS medium employed was a casein peptone starch medium modified from the CPSA medium of Wynn-Williams [11]. The basal medium contained, in 905 ml distilled water, casein hydrolysate, 0.5 g; bacteriological peptone, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; glycerol, 1.0 ml; $FeCl_3$ solution (0.01% w/v), 0.1 ml; and NaCl, 0.1 g. The pH was adjusted to 6.8 and 12 g agar (number 3; Oxoid Limited, Basingstoke, U.K.) added. The medium was autoclaved for 15 min at 121°C. On cooling, filter sterilised cycloheximide solution (0.5% w/v), 10 ml; vitamin solution [13], 5 ml; and trace element solution (consisting of $[g \cdot l^{-1}]$ EDTA, 1.5; $MnSO_4$, 0.5; $FeSO_4$, 0.1; $CaCl_2$, 0.1; $CoCl_2$, 0.1; $ZnSO_4$, 0.1; $CuSO_4$, 0.01; $AlK[SO_4]_2$, 0.01; and Na_2MoO_4, 0.01), 9 ml were added. To complete, autoclaved (15 min at 121°C) K_2HPO_4 solution (1.0% w/v), 20 ml; and soluble starch solution (1.0% w/v), 50 ml were added aseptically.

The oligotrophic medium (OEMS) was based on Bold's basal medium [12]. The basal medium contained 10 ml each of the 1 $g \cdot 400\ ml^{-1}$ solutions of $NaNO_3$, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, K_2HPO_4 and KH_2PO_4 in 936 ml distilled water. To the 986 ml NaCl, 0.1 g; NH_4Cl, 0.173 g; glucose, 5 mg; and arabitol, 5 mg were added with 1 ml each of Bold's EDTA, iron, boron, and micronutrient solutions [12]. The pH was adjusted to 6.8, agar (12 g) added and the medium autoclaved as in the CPS medium. On cooling, filter sterilised cycloheximide solution (0.5% w/v), 10 ml; vitamin B_6 solution (1×10^{-6} $g \cdot l^{-1}$), 25 μl; and vitamin B_{12} solution ($1 \times 10^{-5} g \cdot l^{-1}$), 25 μl were added aseptically.

3.3. Replication of the isolates onto the test media

The oligotrophic and copiotrophic freshwater replication series were replicated from master plates that were chosen from a dilution (10^{-1}) which gave between 20 and 60 well separated colonies on each plate. The colonies on the initial isolation spread plates were replicated onto pre-cooled agar plates of a number of media which contained different organic substrates (Table 1). Replica plating was carried out using sterile velvet material held tightly onto a rubber bung (85 mm diameter) with a large jubilee clip. Prior to replication the pad was autoclaved, dried and cooled to 10°C.

All manipulations were carried out in a laminar flow cabinet (M.D.H. Ltd., Andover, U.K.) and on an ice tray similar to that of Wiebe and Hendricks [14] to minimise contamination and maintain a low temperature. Accurate replication was achieved by placing the velvet pad onto the agar surface of the spread plate and in turn onto each of the pre-cooled replica plates 1 to 11 (Table 1). The first and final replication plates (1 and 11) were the same medium as the master spread plate

Table 1

The media onto which the oligotrophic and copiotrophic series of freshwater bacteria were replicated

Replication Medium Code	OEMS Spread Plates	CPS Spread Plates
1	OEMS	CPS
2	CPS	OEMS
3	FC2	FC2
4	G&M + arabitol	G&M + arabitol
5	G&M + ribitol	G&M + ribitol
6	G&M + glycerol	G&M + glycerol
7	G&M + acetate	G&M + acetate
8	G&M + citrate	G&M + citrate
9	G&M + glucose	G&M + glucose
10	G&M + mannitol	G&M + mannitol
11	OEMS	CPS

to ensure that the bacteria being replicated had remained viable on the velvet pad throughout the replication series.

3.4. Replication media

The FC2 replication medium was modified from that by Tanner [15]. The basal medium contained, in 985 ml distilled water, Na_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; nitrilotriacetic acid, 0.05 g; NH_4Cl, 1.0 g; glycerol, 1.0 g; and NaCl, 0.1 g. The pH was adjusted to 6.8 and agar (12 g) added as in the CPS medium. The medium was autoclaved for 20 min at 115°C. On cooling, filter sterilised cycloheximide solution (0.5% w/v), 10 ml; and 1 ml of each trace element solution: A, ([$g \cdot l^{-1}$] $CaCl_2 \cdot 2H_2O$, 4.0; $MnCl_2 \cdot 4H_2O$, 4.0; $FeCl_3 \cdot 6H_2O$, 2.8); B, ([$g \cdot l^{-1}$] $CoCl_2 \cdot 6H_2O$, 0.2; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2); C, ([$g \cdot l^{-1}$] $ZnSO_4 \cdot 7H_2O$, 0.1; $SrCl_2 \cdot 6H_2O$, 0.2; $NiCl_2 \cdot 6H_2O$, 0.2; H_3BO_3, 0.2; KI, 0.1); and growth factor solution ([$g \cdot l^{-1}$] myo-inositol, 20; pyridoxine HCl, 0.5; calcium pantothenate HCl, 2.0; nicotinic acid, 4.0; thiamine HCl, 0.4; and biotin, 0.001) was added aseptically.

Modified Griffiths and Morita (G&M) medium [10] contained, in 960 ml distilled water, $(NH_4)_2SO_4$, 2 g; KCl, 0.1 g; KH_2PO_4, 0.1 g; K_2HPO_4, 0.5 g; and NaCl, 0.1 g. The pH was adjusted to 6.8 and agar (12 g) added to solidify the medium. The medium was autoclaved for 20 min at 121°C. On cooling, the cycloheximide, vitamin and trace element solutions were added aseptically together with a $MgSO_4 \cdot 7H_2O$ solution (2.4% w/v), 5 ml. Finally, 10 ml of a solution of organic substrate (10% w/v) was added to bring the final carbon source concentration of the medium to 0.1% (1 $g \cdot l^{-1}$). The single carbon sources employed were arabitol, ribitol, glycerol, acetate, citrate, glucose and mannitol.

3.5. Scoring of the growth

The two series of replica plates were incubated for 2.5 weeks at 10°C prior to growth on each organic substrate being scored. Growth was scored only for those colonies for which positive growth was exhibited on plates 1 and 11 of each replication series (Table 1) to ensure the viability of the inoculum throughout the procedure. The growth of these colonies on plates 2 to 10 was recorded simply as growth or no growth, and noted as 1 or 0 respectively. Subjectiveness was reduced by comparing each colony's growth on any replica plate to that on plates 1 and 11.

3.6. Statistical analysis

For each initially-isolated colony, growth on replica plates 2 to 10 was recorded and averaged over the nine replication media to give the 'nutritional flexibility' index,

$$\text{'Nutritional flexibility' index} = \frac{\Sigma G}{n}$$

where ΣG is the number of substrates which supported growth; and n is the number of substrates tested.

The median 'nutritional flexibility' index values for the series of oligotrophic and copiotrophic bacteria were analysed for significant differences using the Mann-Whitney U-test [16]. The null hypothesis of the analysis was that the two series of isolates were drawn from populations having the same ability to utilise the range of substrates tested. A two tailed analysis was carried out and the critical values of the normal deviate of U determined from statistical tables [17].

4. RESULTS AND DISCUSSION

The ability of each bacterial isolate to grow on each of the organic substrates tested, and their

Table 2

The ability of copiotrophic and oligotrophic freshwater bacteria to grow on the replication media. Growth is denoted by a value of 1 and no growth by 0

Isolate	Replication Medium Code									Number of substrates that supported growth	'Nutritional flexibility' index
	2	3	4	5	6	7	8	9	10		
Copiotrophic isolates											
1	0	0	0	0	0	0	0	0	0	0	0.000
2	0	0	0	0	0	0	0	0	0	0	0.000
3	0	1	0	0	1	0	0	0	0	2	0.222
4	0	0	0	0	0	0	0	0	0	0	0.000
5	0	0	0	1	0	0	0	0	1	2	0.222
6	0	0	0	0	0	0	0	0	0	0	0.000
7	0	0	0	0	0	0	0	0	0	0	0.000
8	0	0	0	0	0	0	0	0	0	0	0.000
9	0	1	0	0	1	1	1	1	0	5	0.556
10	0	1	1	0	1	1	1	1	1	7	0.778
11	0	0	0	0	0	0	0	0	0	0	0.000
12	0	0	0	0	0	0	0	0	0	0	0.000
13	1	1	1	1	1	0	1	1	1	8	0.889
14	0	0	0	0	1	1	1	1	0	4	0.444
15	0	0	1	0	1	1	1	1	0	5	0.556
16	0	0	0	1	1	1	1	1	0	5	0.556
17	0	0	0	0	0	0	0	0	0	0	0.000
18	0	0	0	0	1	0	0	1	0	2	0.222
19	0	1	0	0	0	0	1	0	0	2	0.222
20	0	0	0	0	0	0	1	0	0	1	0.111
21	0	0	0	0	0	0	0	0	0	0	0.000
22	0	0	0	0	0	0	0	0	0	0	0.000
23	0	0	0	0	0	0	0	0	0	0	0.000
24	0	0	0	0	0	0	0	0	0	0	0.000
25	0	1	1	1	1	1	1	1	0	7	0.778
26	1	1	1	1	1	1	1	1	1	9	1.000
27	0	1	1	1	1	1	1	1	1	8	0.889
28	1	1	1	1	1	1	1	1	0	8	0.889
29	0	0	0	1	1	0	1	1	0	4	0.444
30	0	1	1	1	1	0	1	1	0	6	0.667
31	0	0	0	0	0	0	0	0	0	0	0.000
Oligotrophic isolates											
1	1	0	0	0	0	0	0	0	0	1	0.111
2	1	1	1	1	1	0	1	1	1	8	0.889
3	1	1	1	1	1	1	1	1	1	9	1.000
4	1	1	1	1	1	0	1	1	1	8	0.889
5	0	1	1	1	1	1	1	1	1	8	0.889
6	0	1	1	1	1	1	1	1	1	8	0.889
7	0	1	1	1	1	1	1	1	1	8	0.889
8	0	1	1	1	1	0	1	1	1	7	0.778
9	1	1	1	1	1	1	1	1	1	9	1.000
10	0	1	1	1	1	1	0	1	1	7	0.778
11	1	1	1	1	1	1	0	1	1	8	0.889
12	1	1	1	1	1	1	0	1	1	8	0.889
13	1	1	1	1	1	0	0	1	1	7	0.778
14	1	1	1	1	1	0	0	1	1	7	0.778

Table 3

The median 'nutritional flexibility' indices of the copiotrophic and oligotrophic series of freshwater bacteria

Isolate series	Sample size	Median 'nutritional flexibility' index	Interquartile range
Copiotrophs	31	0.222	0.000 to 0.556
Oligotrophs	14	0.889	0.778 to 0.889

'nutritional flexibility' indices, are shown in Table 2. The median 'nutritional flexibility' index values for the oligotrophic and copiotrophic bacteria were calculated and are shown in Table 3. The microorganisms isolated on an 'oligotrophic' medium had a significantly higher 'nutritional flexibility' index than those isolated on a 'copiotrophic' medium. The amount of assimilable organic carbon present in the agar used in the media was unknown, and it is possible that the OEMS medium was not 'oligotrophic' in the strict terms described by Kuznetsov et al. [1]. However, the 'oligotrophic' medium did have less organic carbon than the 'copiotrophic' medium.

Five out of the fourteen oligotrophic isolates were shown to be unable to grow on the copiotrophic CPS medium (Table 2). This may suggest that they were obligate oligotrophs, although Martin and MacLeod [5] have cautioned that the ability to use high or low concentrations of substrates may depend upon the substrate, or mixture of substrates, available. They showed that an apparently obligate oligotroph was inhibited by high concentrations of succinate and aspartate. Inhibition at high peptone concentrations was due to the presence of these inhibitors, and not to the presence of high concentration of organic carbon per se. Hattori [18] has also demonstrated that the ability of oligotrophic soil microorganisms to utilise a substrate may be influenced by the presence either of different amino acids or by changes of concentrations of inorganic salts.

Our data show that bacteria isolated on a low nutrient oligotrophic medium from a freshwater Antarctic lake (Heywood Lake) could utilise a significantly broader range of organic carbon sources than those isolated on a richer copiotrophic medium ($P < 0.0001$). Harder and Dijkhuizen [19] have pointed out that in microorganisms from oligotrophic environments there tends to be an increase in the affinity of uptake systems for substrates; while there is a decrease in the substrate specificity of these uptake systems. It has also been demonstrated [20] that the presence of multiple substrates may influence the uptake kinetics of individual substrates. These may all be adaptations to low energy habitats where the ability to utilise, possibly simultaneously, a wide range of organic substrates may be of survival value. Our findings tend to confirm Poindexter's hypothesis that oligotrophic bacteria are more nutritionally flexible than copiotrophic bacteria.

Horowitz et al. [21] have previously reported an investigation into the versatility of oligotrophs to different carbon sources. Plates of different nutritional composition, with final substrate concentrations between 0.1 and 0.15% (w/v), were inoculated with bacteria previously isolated on low and high nutrient media from Cook Inlet, Alaska. The presence of growth was observed to assess the nutritional versatility of the isolates. The bacteria isolated on low nutrient media were found to be nutritionally far more versatile than those isolated on high nutrient media, having significantly higher 'nutritional utilization indices' for alcohols, carboxylic acids, amino acids and hydrocarbon substrates. No significant difference was shown for the ability to utilise a number of carbohydrates. The data obtained in the present study therefore corroborates the findings of Horowitz et al. Both studies are consistent with the hypothesis of Poindexter [2] that bacteria adapted to oligotrophic environments will be more nutritionally flexible than bacteria adapted to copiotrophic environments.

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Distinction of emigration by telotroch formation and death by predation in peritrich ciliates: SEM observations on the remaining stalk ends

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1. SUMMARY

Scanning electron microscope (SEM) observations of remaining stalk ends were made to distinguish emigration by telotroch formation and death by predation in sessile peritrich ciliates (protozoa). *Carchesium polypinum* had nail head-like stalk ends after the formation of telotrochs and *Epistylis* sp. had cone-shaped stalk ends. When the peritrichs were preyed upon by *Chaetogaster diastrophus* (Oligochaeta) or *Eosphora anthadis* (Rotifera) the stalks remaining showed jagged ends. From these results emigration could be detected by the nail head-shaped stalk ends for *C. polypinum* and the cone shapes for *Epistylis* sp. Predation could be detected by the remaining jagged stalk ends.

2. INTRODUCTION

Sessile peritrich ciliates are often abundant in streams and their population densities are known to fluctuate greatly [1]. By tracing the attachment and development of each colony, we estimated the colonization rates and in situ growth rates of the colony-forming peritrich *Carchesium polypinum* in an urban river [2]. The rapid population increases were caused mainly by high growth rates at the site along with some increase due to immigration. Survival rates of colonies for colonial species and of individuals for solitary species could also be estimated by tracing the fate of each colony or individual. Small colonies and solitary species were sometimes subject to heavy losses. Up to 75% of the freshly attached *Vorticella microstoma* individuals had disappeared by the next day. However, the cause of the disappearances could not be determined.

Sessile peritrichs are known to form telotrochs (free swimming swarmers) and swim away when under stress (e.g., lack of food or oxygen). They are also readily preyed upon by the oligochaete *Chaetogaster* [3,4] and by carnivorous ciliates [5,6]. Whether the cells actively detach or are preyed upon, the stalks are left behind.

In the present study an attempt to distinguish the loss of individuals due to telotroch formation and death by predation was made by scanning electron microscope (SEM) observations on the remaining stubs of the peritrich stalks.

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3. MATERIALS AND METHODS

Two peritrich species, *Carchesium polypinum* and *Epistylis* sp., were isolated from the Yazawa River, a small urban river polluted by domestic wastes. They were cultured in the laboratory and identified according to Kudo [7], Curds [8] and Bick [9].

The predators used in this study were the oligochaete, *Chaetogaster diastrophus,* and the rotifer, *Eosphora anthadis,* which were both observed preying on peritrich ciliates in the Yazawa River. The rotifer was identified by Dr. W. Koste and the oligochaete by Dr. A. Ohtaka. Stock cultures of *Chaetogaster* were maintained on a mixed diet of small ciliates and rotifers. *Eosphora* were fed on *Epistylis.*

Culture medium for the peritrichs was prepared by boiling 0.03% (w/v) cereal leaves (Sigma Chem. Co.) in Prescott James Solution [10] for 15 min and filtering through filter paper. The bacterium *Enterobacter aerogenes* was inoculated into the autoclaved medium as food for the peritrichs a day before the start of the experiments. In the stock cultures, the peritrichs often form colonies attached upside down to the medium surface. As these colonies readily become attached to solid substrates as soon as they are encountered, it was possible to transfer the peritrichs to transparent plastic films simply by touching the medium surface with the films. The films with *C. polypinum* or *Epistylis* sp. were placed in 6 cm diameter petri dishes with fresh medium and cultured at 25°C for a day or two. Before telotrochs were formed, the predators were added separately to the dishes to feed on the peritrichs. *Chaetogaster* was added at a density of 10 per dish and cultured for 2 days, while 20 to 30 *Eosphora* were added to a dish and cultured for 1 day. During this process, the feeding behavior of the predators was also observed. The samples were then fixed with 2% glutaraldehyde and stored in a refrigerator. As control, the peritrich colonies on the plastic films were cultured without changing the medium for up to a week. After the formation of telotrochs was confirmed, the films were fixed as above.

For SEM observations the fixed samples were dehydrated by gradually increasing the concentration of ethanol followed by replacement with isoamyl acetate. As the fragility of the stalks increased with dehydration, the samples were never lifted above the liquid surface until the dehydration was completed. The samples were then critical point dried with CO_2, mounted on aluminum stubs and coated with gold. The ends of the peritrich stalks were examined with a scanning electron microscope (Akashi Beam Technology, WS-250).

The shape of each stalk end was categorized and noted. Stalk ends which were overlapping or obscured by bacteria and detritus were listed as indistinguishable. The number of stalks with intact cells still attached was also counted.

Statistical analysis of the effect of predators on the shapes of the stalk ends was made by chi-square tests.

4. RESULTS

Chaetogaster diastrophus searches for prey by crawling on the surface of the substrates while moving its head from side to side as in Taylor's observations of *Chaetogaster langi* [3]. When this oligochaete encounters a peritrich cell it immediately seizes the cell in its mouth, then tries to break the stalk by pulling or wriggling its body. Sometimes the stalks do not break, and the oligochaete then expels its prey (the rejected prey are seldom injured and usually resume normal activities).

Eosphora searches for prey by swimming near the surface of the substrate. Once a prey colony is encountered, the rotifer attaches itself to it by excreting a mucus-like substance and feeds on the peritrichs by nibbling each cell one by one. As long as there is food, the rotifer spends its entire life on the peritrich colony. Even the eggs are laid on the peritrich stalks.

The stalks of both *Carchesium polypinum* and *Epistylis* sp. were observed to be widened at the point of attachment to the cells, as reported by Zagon [11], forming nail head-like structures (Figs. 1 and 2). On telotroch formation, a band of cilia (the posterior ciliary wreath) appeared at the aboral region of the cell (Fig. 3), then the zooids

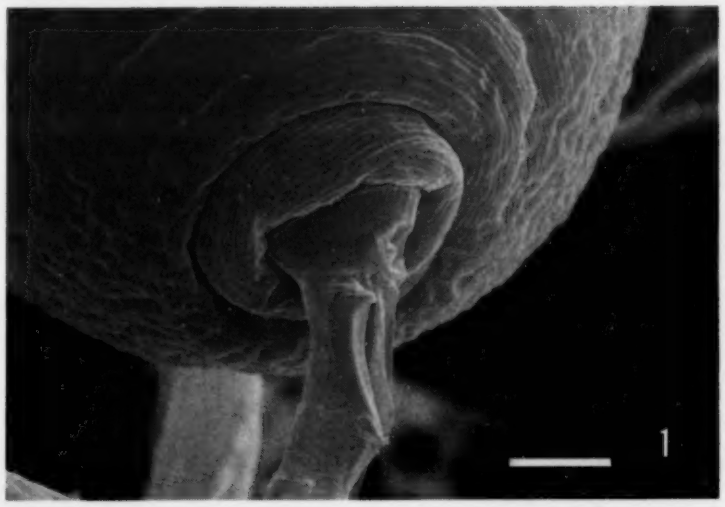

Fig. 1. SEM photograph of the scopula region (base) of *Carchesium polypinum*. The width of the stalk increases forming nail head-like structures (bars indicate 5 μm).

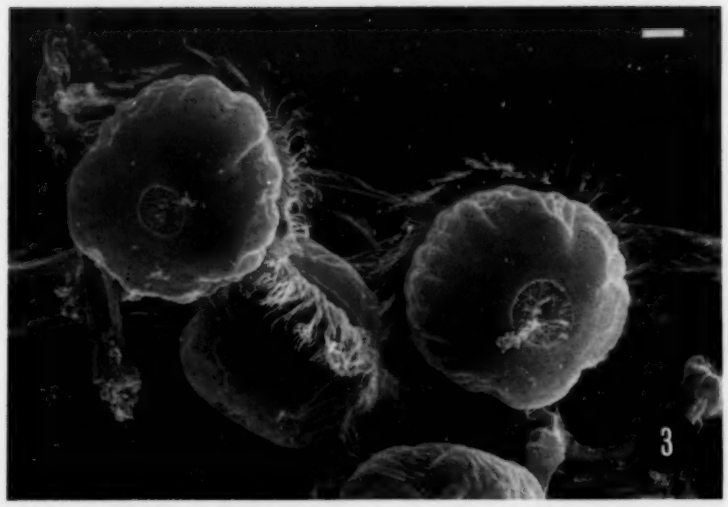

Fig. 3. Telotroch formation in *Carchesium polypinum*. The development of posterior ciliary wreaths can be seen in the aboral region.

became detached from the stalks and swam away. In the control series, in which the peritrichs had formed telotrochs, most of the remaining stalk ends showed the nail head-like structures for *C. polypinum* (Fig. 4) while the stalk ends of *Epistylis* sp. had a cone-like structure (Fig. 5).

In the series in which *Eosphora anthadis* was added, the number of stalks with jagged ends increased in both *C. polypinum* and *Epistylis* sp. (Figs. 6 and 7). In the series with *Chaetogaster* the number of stalks with jagged ends also increased for both peritrichs. In the observations made so far, no noticeable difference could be detected between the cells consumed by either predator.

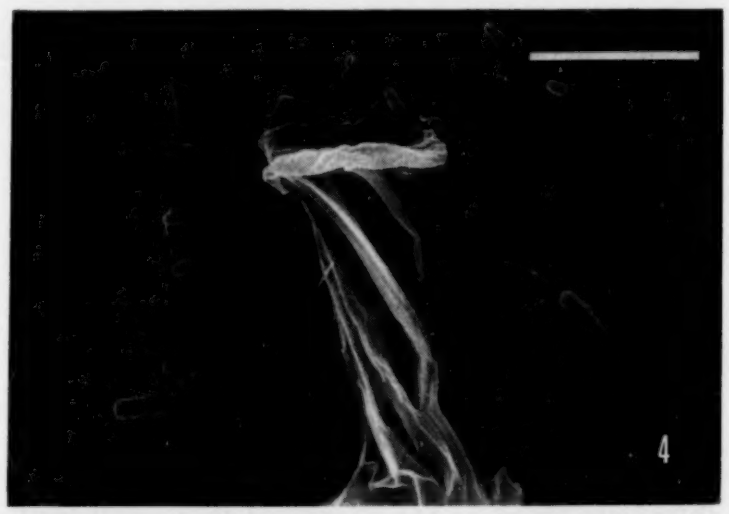

Fig. 4. Nail head-like remains of *Carchesium polypinum* stalks after telotroch formation.

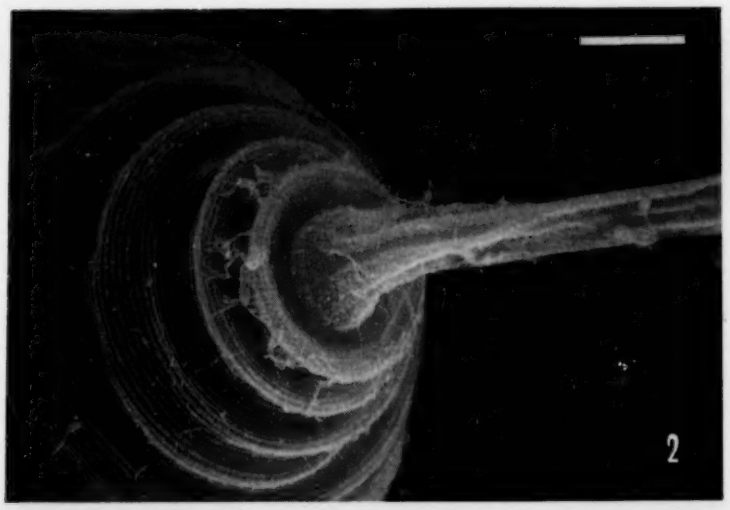

Fig. 2. Scopula region of *Epistylis* sp. The nail head-like structure similar to those in *Carchesium polypinum* can be observed.

Fig. 5. Cone-shaped remains of stalks of *Epistylis* sp. after telotroch formation.

Table 1

Number and frequency of stalk end types observed

Peritrich	Predator	Type of stalk end					Intact cells	Significance
		Cone	Nail head	Jagged	Flat	Indist.		
Epistylis	None (control)	321	12	23	14	43	33	
(%)		86.8	3.2	6.2	3.8			
	Eosphora	2	18	87	8	61	0	$P < 0.01$
		1.7	15.7	75.7	6.9			
	Chaetogaster	9	75	116	1	105	59	$P < 0.01$
		4.5	37.3	57.7	0.5			
Carchesium	None (control)	1	254	57	3	114	260	
(%)		0.3	80.6	18.1	1.0			
	Eosphora	1	38	182	10	127	2	$P < 0.01$ *
		0.4	16.5	78.8	4.3			
	Chaetogaster	0	66	131	0	37	365	$P < 0.01$ *
		0	33.5	66.5	0			

(%): Frequency of stalk end types of the distinguished stalk ends.
Indist.: Indistinguishable.
Significance: Difference from control by chi-square tests.
* As the number of cones and flats was very low, the tests were made using only nail heads and jaggeds.

Some stalk ends could not be characterised because of masking by bacteria and/or filamentous matter (Fig. 8). The filamentous matter was probably mucus that the rotifer excreted when they attached to the peritrich stalks. A small number of stalk ends (especially in *Epistylis*) showed a smooth flat surface.

The number and frequency of the remaining stalk end types that could be distinguished are shown in Table 1. The shapes of these stalk ends were categorized into 4 types; cone, nail head, jagged and flat. Frequency distribution of stalk end types of both peritrich species changed significantly ($P < 0.01$) when the predators were introduced. In the *Epistylis* series, the frequency of cone ends dropped from 86.8 to 1.7%, while the jagged ends increased from 6.2 to 75.7% when *Eosphora* was added. With the addition of *Chaetogaster* the cones dropped to 4.5% and the jagged ends increased to 57.7%. There was also an increase in the frequency of the nail head stalk ends. In the *C. polypinum* series 80.6% of the control

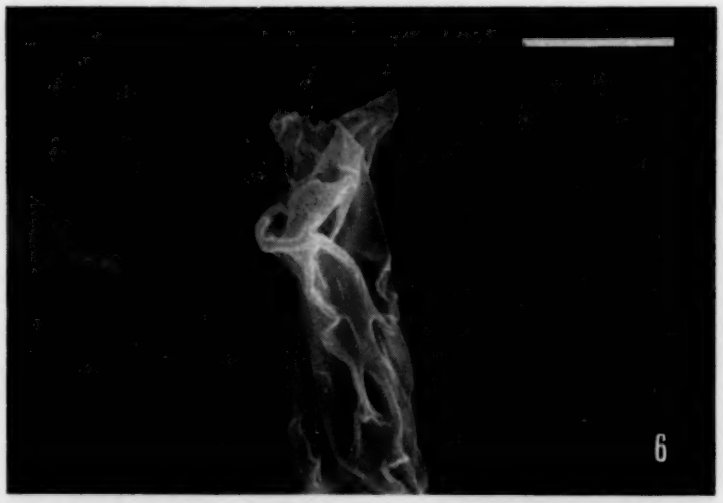

Fig. 6. Jagged stalk ends of *Carchesium polypinum* after predation by *Eosphora anthadis*.

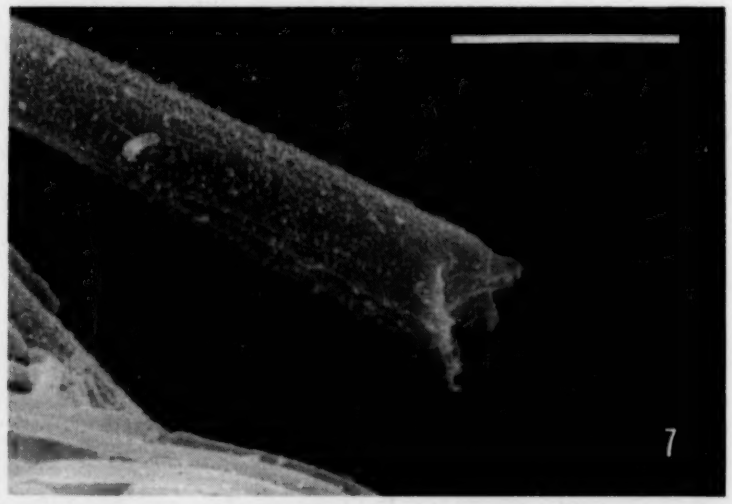

Fig. 7. Jagged stalk ends of *Epistylis* sp. after predation by *Eosphora anthadis*.

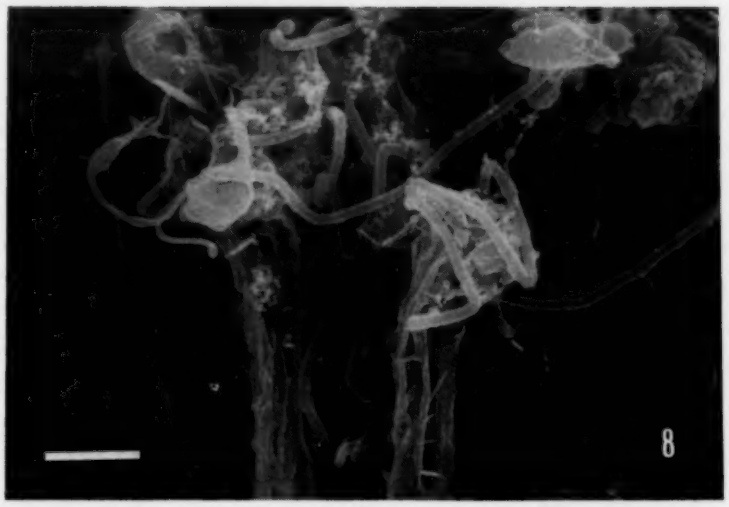

Fig. 8. Stalk ends of *Epistylis* sp. covered with bacteria and filamentous matter.

had nail head ends while 18.1% were jagged. With the addition of the predators, the jagged ends increased to 78.8% with *Eosphora* and 66.5% with *Chaetogaster.*

5. DISCUSSION

The shapes of the remaining stalk ends of both *Carchesium polypinum* and *Epistylis* sp. were significantly influenced by the presence of the predators, *Chaetogaster diastrophus* and *Eosphora anthadis.* Since the cone-shaped end for *Epistylis* sp. and the nail head-like end for *C. polypinum* dominated the control series, these stalk end shapes were considered to be the result of telotroch formation. In the control series a few stalks showed jagged ends in both peritrichs, probably due to stalk breakage during the dehydrating and critical point drying process. The jagged shaped stalk ends dominated the series with predators, so these ends can be regarded as indicating predation.

The frequency of jagged ends was higher in the presence of *Eosphora* for both peritrich species (*Epistylis* 75.7%, *C. polypinum* 78.8%) than with *Chaetogaster* (*Epistylis* 57.7%, *C. polypinum* 66.5%). Two reasons for this difference can be deduced from the behavior of these predators. *Chaetogaster diastrophus* in its search for prey sometimes passes under large colonies without disturbing them. The large number of intact cells remaining in the *Chaetogaster* series for both peritrich species is probably a result of this (Table 1). Since the cells at the top of the colonies are left alone, they have a chance to produce telotrochs thus leading to the decrease in the frequency of jagged ends. *Eosphora* usually stays attached to the colonies of its prey until all the cells are eaten. This leads to the very large decrease in the number of intact cells remaining in the series with the rotifer (Table 1).

Another reason for the differences in the results with the two predators is that *Chaetogaster* first seizes its prey then tries to break the stalk by pulling. The stalks of *Epistylis* are rigid and do not easily break. There is a possibility that sometimes the cells break off at the base of the cell (scopula) (Fig. 2) leaving the nail head like structures. This is probably the cause of the increase in the proportion of the nail head ends when *Chaetogaster* was added to *Epistylis*. Because of this, it might be better to consider the nail head ends of *Epistylis* sp. as being due to the predator. The stalks of *C. polypinum* are more fragile and easily broken when attacked by *Chaetogaster*, but the possibility still remains that some of the nail head shaped stalk ends might actually have been due to the predator in this case too.

When the rotifer *Eosphora* feeds, it chews the prey cell from the top down to the stalks so that no more nail head-like structures remain. This results in the high frequency of jagged stalk ends. A small proportion of the stalks showed flat ends (Table 1), a shape which remains to be explained.

From these results it can be concluded that by observing the remaining stalk stubs, telotroch formation can be distinguished from death by predation. If this method is applied in the field, it should be possible to estimate emigration due to telotroch formation and mortality by predation at the site. In the field, bacteria and detritus quickly become attached to the stalk stubs making the shapes difficult to distinguish. However, there is an advantage in this, because the frequency of the distinguished stalk forms will indicate how recently predation or emigration has occurred.

Schönborn [4] reported that *Chaetogaster diastrophus* consumes 72% of the annual ciliate production in the River Saale. In the Yazawa River

where this oligochaete was collected, densities of up to 10 individuals $\cdot$ cm^{-2} have been observed. With the present method the direct effect of predation on the peritrich communities can be estimated.

By combining the method described here with that of tracing the growth and fate of colonies and individuals [2], it should be possible to estimate the actual growth, death, immigration and emigration of sessile peritrichs in the field. Factors affecting the population dynamics of the peritrichs can be analyzed and the role of the peritrichs in their environments can be closely studied.

ACKNOWLEDGEMENTS

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Reduction of tetrazolium salts by sulfate-reducing bacteria

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1. SUMMARY

The reduction of tetrazolium salts by the sulfate-reducing bacteria, *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis,* was examined. *D. desulfuricans* and *D. orientis* reduced triphenyltetrazolium chloride (TTC) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) forming intracellular formazan deposits. The reduction rate of INT was higher than that of TTC. INT reduction was not inhibited by the addition of sulfate or molybdate, and sulfate uptake was inhibited by the addition of both INT and molybdate. The ratio of intracellular formazan forming cells to acridine orange direct counts in both strains decreased with culture age and starvation time.

2. INTRODUCTION

Aerobic bacteria can reduce tetrazolium salts, at specific sites of their electron transport system (ETS), to formazan which accumulates intracellularly as an optically dense deposit. This reaction has been used for the measurement of ETS activity [1–8] and for distinguishing respiring from nonrespiring (inactive) cells in various environments [9–13]. These methods have been infrequently applied to anaerobic bacteria. Recently, Oren [14] examined the potential of tetrazolium reduction by various anaerobic bacteria. He reported the inability to reduce tetrazolium salts of a sulfate-reducing bacterium (SRB), *Desulfovibrio* sp., which was isolated from a freshwater pond.

However, we found that *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis* could reduce two tetrazolium salts, triphenyltetrazolium chloride (TTC) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT). The aim of the present study is first to show the evidences that SRB can reduce tetrazolium salts, secondly to develop a method to measure ETS activity of SRB, and thirdly to examine successive changes of the proportion of respiring cells to total cell counts during starvation.

3. MATERIALS AND METHODS

3.1. Organisms and cultivation

Two strains of SRB, *Desulfovibrio desulfuricans* (IFO 13692) and *Desulfotomaculum orientis*

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(ATCC 19365), were used in this study. The growth medium was the C medium of Butlin et al. [15]. The C medium contained (g/l) K_2HPO_4 (0.5), NH_4Cl (1), Na_2SO_4 (1), $CaCl_2 \cdot 2H_2O$ (0.1), $MgSO_4 \cdot 7H_2O$ (2), Na-lactate (3.5), $FeSO_4 \cdot 7H_2O$ (0.2), and yeast extract (Difco, U.S.A.) (1) (pH 7.2). The autoclaved medium was supplemented with a separately autoclaved reducing agent (0.045% sodium thioglycolate). SRB strains were precultured on the C medium for 4 days at 30 °C. Ten ml of the culture was inoculated into 1 litre medium bottles filled with the C medium, which was modified by reducing the amount of $FeSO_4 \cdot 7H_2O$ to trace. The bottles were sealed with screw caps and incubated at 30 °C in the dark. From preliminary experiments, cultures of first 3 days, 3 to 8th day and over 8th day in this culture condition corresponded to those in exponential, stationary and death phases for both strains, respectively. After appropriate incubation time, the cells were harvested by centrifugation at 6000 rpm and 4 °C for 25 min and washed twice with anaerobic phosphate buffer (KH_2PO_4, 0.11 g/l; $Na_2HPO_4 \cdot 12H_2O$, 0.64 g/l; pH, 7.0) while flushing with N_2 gas. The cell pellets were then resuspended in 10 ml of anaerobic phosphate buffer under N_2 gas.

The assay mixture was prepared as follows. A portion (0.5–1.0 ml) of the cell suspension was inoculated into a 120 ml serum vial containing 50 ml of the medium (Ingvorsen and Jørgensen [16]) devoid of sulfate and lactate (IG) and then sealed with a butyl rubber stopper after bubbling with N_2 gas. The IG medium contained (g/l) KH_2PO_4 (0.11), $Na_2HPO_4 \cdot 12H_2O$ (0.64), NaCl (1.0), KCl (0.5), $MgCl_2 \cdot 6H_2O$ (0.6), $CaCl_2 \cdot 2H_2O$ (0.2), NH_4Cl (0.25), 1 ml trace element solution, and 5 ml vitamin solution (pH, 7.0). The trace element solution [17] contained (mg/l) $FeCl_2 \cdot 4H_2O$ (1500), H_3BO_3 (60), $MnCl_2 \cdot 4H_2O$ (100), $CoCl_2 \cdot 6H_2O$ (120), $ZnCl_2$ (70), $NiCl_2 \cdot 6H_2O$ (25), $CuCl_2 \cdot 2H_2O$ (15), $Na_2MoO_4 \cdot 2H_2O$ (25), and 6.5 ml HCl (25%). The vitamin solution [18] contained (mg/l) biotin (2), nicotinic acid (20), *p*-aminobenzoic acid (10), thiamin (20), panthotenic acid (10), pyridoxamin (50), and cobalamin (10).

3.2. Reduction of tetrazolium salts by SRB

The assay mixture (50 ml) as prepared above was preincubated for 60 min at 30 °C and then oxygen-free lactate (final concentration, 30 mM) and 5 ml of 0.2% INT or TTC (Sigma Co., U.S.A.) oxygen-free aqueous solution [9] were added through the rubber stopper. Subsamples were periodically withdrawn by a syringe. The reaction was stopped with glutaraldehyde (final concentration, 1.0% w/w). Formazan was determined according to the method of Oren [14] modified as follows. In extracting formazan in cells, 0.5 ml of the sample was mixed with 1 ml of *n*-amylalcohol, sonicated at 40 W for 3 min, and centrifuged. Sonication was essential to extract the intracellular formazan deposits of the SRB. The light absorbance of the alcohol layer was measured at 485 nm using a spectrophotometer (Shimadzu, UV-160, Japan). The concentration of formazan was estimated from a standard curve of INT-formazan or TTC-formazan reduced by sodium dithionite.

To examine the effect of Na_2MoO_4 (final concentration, 20 mM) or Na_2SO_4 (final concentration, 15 mM) on INT reduction by SRB, the anaerobic reagents were injected through the rubber stoppers before the addition of INT.

3.3. Sulfate uptake by SRB

To evaluate the effects of inhibitors on sulfate reduction, ^{35}S-sulfate uptake experiments were carried out according to Ingvorsen and Jørgensen [16]. After addition of anaerobic lactate solution (final 30 mM), the assay mixture prepared above was preincubated at 30 °C for 1 h. Anaerobic solutions of non-radioactive sulfate (final 15 mM) or radioactive sulfate (10μCi, Amersham Japan) were injected through the rubber stopper. During the incubation at 30 °C, aliquots (0.75 ml) were periodically withdrawn by a syringe and transferred into a 1.5 ml micro test tube (Eppendorf, F.R.G.) containing anaerobic alkaline zinc acetate solution (0.5 ml, 1 M). The mixture was centrifuged after vigorous mixing. One ml of the cell-free supernatant was mixed with 10 ml of Scintisol (Dotite, Japan). The radioactivity of residual ^{35}S-sulfate in the supernatant was counted with a liquid scintillation counter (Beckman LS3801, U.S.A.). Corrections for quenching were made by the external standard channels ratio method.

To examine the effect of INT or Na_2MoO_4 on

sulfate uptake by SRB, the anaerobic reagents were injected through the rubber stoppers before the addition of sulfate.

3.4. Starvation experiments

Cell suspensions were prepared from a culture in exponential growth phase (2 days cultivation) as described above (3.1). A half ml of these cell suspensions was inoculated in 50 ml of anaerobic vitamin- and trace element-free IG medium in a 120 ml serum vial and starved at 30°C. Total count, plate count and the number of cells having intracellular formazan deposits were enumerated at appropriate time intervals during starvation. To count the number of cells having intracellular formazan deposits, INT solution was injected through the rubber stopper after additions of the vitamin solution, the trace element solution, and lactate at the same concentrations as described above.

3.5. Enumeration of bacteria

Total counts (TC) in the samples were obtained by the method of Hobbie et al. [19] modified by Konda [20] with acridine orange (Merck, F.R.G., 0.1%) using an epifluorescent microscope (Nikon, Japan).

Cells containing intracellular formazan deposits were enumerated using a phase-contrast microscope (Nikon).

Plate counts (PC) were obtained by the anaerobic Petri dish method of Wakao and Furusaka [21]. The enumeration medium was the C medium containing agar (15 g/l). The medium was inoculated with the sample, appropriately diluted with sterilized distilled water. Colonies were enumerated by the naked eye at appropriate time intervals during the incubation period at 30°C. The plate count data were applied to the first order reaction (FOR) model proposed by Hattori [22,23]. The value of PC was expressed by the parameter $N\infty$ (the expected number of colonies on a plate at infinite incubation time) of the model estimated by the method of Ishikuri et al. [24] using five replicate plates.

4. RESULTS

4.1. Formation of intracellular formazan deposits by SRB

D. desulfuricans and *D. orientis* reduced INT and TTC to formazan deposits in their cells, as seen by phase contrast microscopy (1500×, Fig. 1). Formazan was not produced under the same conditions when SRB were killed by glutaraldehyde (final 1%) before addition of tetrazolium salts. The cells with intracellular formazan deposits were termed 'formazan forming cells' (FFC).

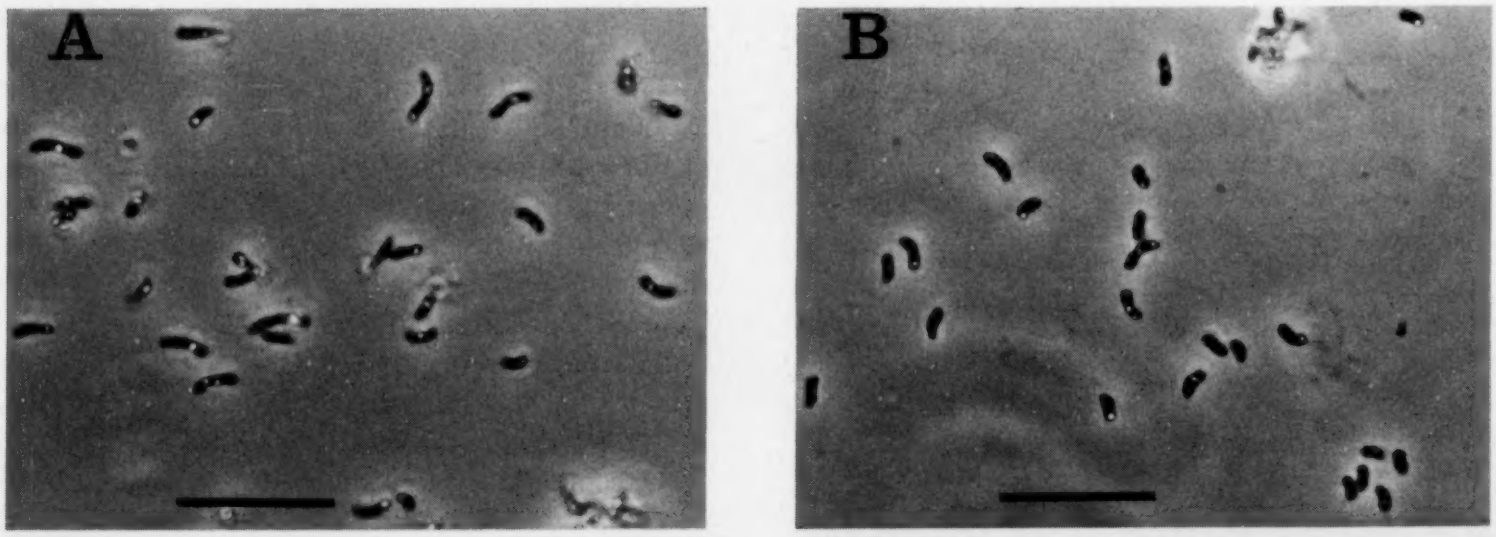

Fig. 1. Intracellular formazan deposits of *Desulfovibrio desulfuricans* (A) and *Desulfotomaculum orientis* (B). Scale bars are 10 μm.

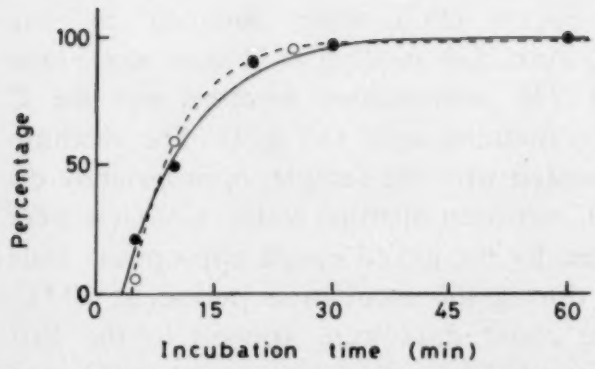

Fig. 2. Effect of the incubation time on formazan formation by *Desulfovibrio desulfuricans* (●) and *Desulfotomaculum orientis* (○). The total counts were arbitrarily taken as 100. The percentage indicates the proportion of formazan forming cells to total counts.

The presence of microscopically detectable formazan deposits was determined after different incubation times. Fig. 2 shows the relationship between the ratio of FFC to TC and incubation time. The percentages for *D. desulfuricans* and *D. orientis* were 97.8 and 99.0 after 30 min, respectively. In subsequent experiments, FFC were enumerated after 60 min of incubation.

As the number of formazan-positive cells increased, there was a simultaneous increase in formazan concentration (Fig. 3). The reduction rate of INT was higher than that of TTC for both strains. Addition of the terminal electron acceptor, sulfate (final concentration, 30 mM), had no effect on the reduction rate of INT.

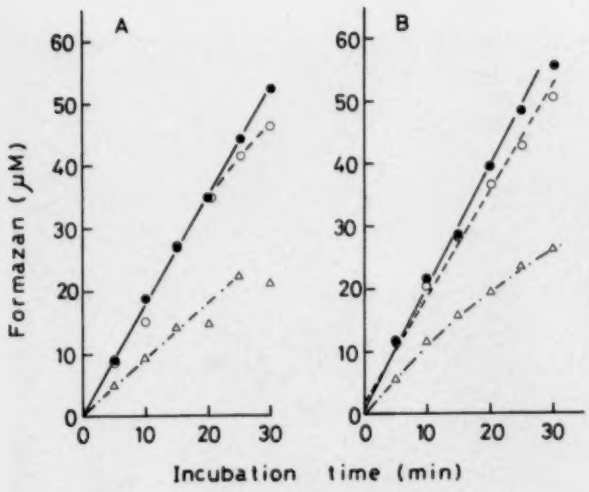

Fig. 3. The production of formazan from INT (●, ○) and TTC (△) by *Desulfovibrio desulfuricans* (A) and *Desulfotomaculum orientis* (B). (●) indicates addition of sulfate.

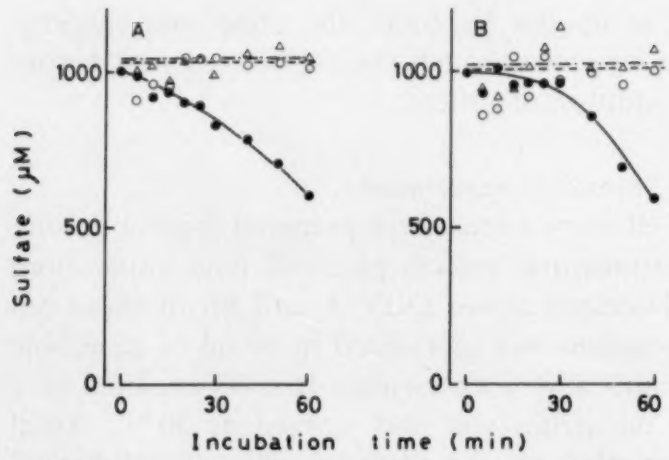

Fig. 4. Effect of the addition of INT (○) or Na_2MoO_4 (△) on sulfate uptake by *Desulfovibrio desulfuricans* (A) and *Desulfotomaculum orientis* (B). (●) indicates no addition of INT and Na_2MoO_4.

Sodium molybdate, another specific inhibitor of sulfate reduction [25], did not inhibit INT reduction by either strain. Sodium molybdate and INT, however, both inhibited sulfate uptake by the test strains (Fig. 4).

ETS activities were measured at different incubation temperatures ranging from 8°C to 37°C. The linear relationships were obtained between the reciprocal of the absolute temperatures and the log values of the activities (increase rates of absorbance at 485 nm/ml/15 min) for both strains. The activation energy of INT reduction, μ, of *D. desulfuricans* and *D. orientis*, was estimated from the slope to be 17.7 and 16.6 kcal/mol, respectively.

4.2. Effects of culture age or starvation on FFC of SRB

The ratio of PC or FFC to TC for SRB in the exponential growth phase was compared with those in the stationary and death phases (Table 1). The percentages of FFC to TC for *D. desulfuricans* were 89.7% in the stationary phase and 18.4% in the death phase. Similarly, those of *D. orientis* were 87.7% and 3.2%, respectively. The PCs of both strains were smaller than FFCs in every growth phase. Thus, the proportion of FFC and PC to TC decreased with the culture age.

Effects of starvation on the ratio of FFC to TC in *D. desulfuricans* and *D. orientis* were examined. Fig. 5 shows TC, FFC, and PC of both strains

Table 1

Comparison among total counts, the number of formazan forming cells, and plate counts of *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis* in different cultivation times

Strain	Cultivation time (days)	Plate counts (%)[1]	Formazan forming cells (%)	Total counts (cells/ml)
Desulfovibrio desulfuricans	2	40.2 ±4.070	98.2±2.151	$7.69\pm2.082\times10^{8}$
	5	44.9 ±8.215	89.7±4.897	$1.14\pm0.177\times10^{9}$
	10	ND[2]	18.4±2.791	$1.90\pm0.601\times10^{9}$
Desulfotomaculum orientis	2	42.5 ±6.963	99.3±1.450	$1.60\pm0.300\times10^{8}$
	8	9.80 ±0.7773	87.7±4.010	$2.98\pm0.154\times10^{9}$
	15	0.244±0.00938	3.2±1.344	$2.21\pm0.355\times10^{9}$

[1] Total count was arbitrarily taken as 100.
[2] Not determined.

during starvation. The PC decreased exponentially with time immediately after starvation in both strains, although the TC did not show a drastic change throughout the experimental period. On the other hand, the FFC did not begin to decrease until 100 h. After 300 h of starvation, the differences among each count became larger (TC > FFC > PC): The proportions of FFC and PC to TC were 3.7% and 0.0088%, respectively, in *D. desulfuricans,* and 19.8% and 0.10%, respectively, in *D. orientis*. The starvation resulted in the increase of the ratio of respiring, but not in the ratio of growing cells to TC.

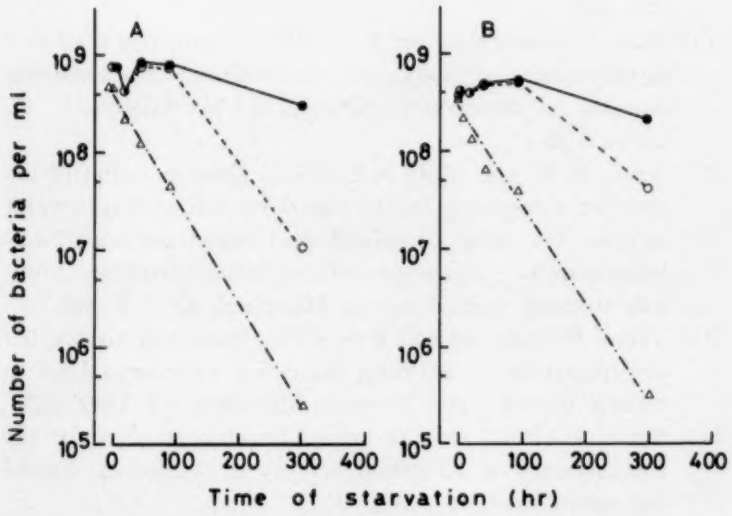

Fig. 5. Comparison of total counts (●), the numbers of formazan forming cells (○), and plate counts (△) of *Desulfovibrio desulfuricans* (A) and *Desulfotomaculum orientis* (B) during starvation.

5. DISCUSSION

Oren [14] reported that *Desulfovibrio* sp. isolated from a pond did not reduce tetrazolium salts at a significant rate. However, our results (4.1 and 4.2) clearly showed that *D. desulfuricans* and *D. orientis* reduced INT and TTC and produced intracellular formazan deposits. Thus, formazan formation can be used to measure ETS activity of both strains. Reduction rates of INT by both strains were higher than that of TTC. This result agrees with the findings by Trevors [26] that in soils INT was reduced more than TTC. It is concluded that INT is a better substrate than TTC for the measurement of ETS.

SRB have electron transport chains involved in the regeneration of NAD^+ from NADH by the reduction of thiosulfate to sulfide. Kenner and Ahmed [27] suggested that in diatoms and green algae the INT reduction site was at the level or near that of the cytochrome *b*-ubiquinon complex. The INT reduction by SRB may be supported physiologically by the presence of cytochrome c_3 in *Desulfovibrio* [28,29] and by cytochrome *b* in *Desulfotomaculum* [30] in their electron transport chains. Inhibition of sulfate reduction by INT may be thus explained.

The activation energy (kcal/mol) for sulfate reduction in various aquatic sediments ranged from 13.0 to 20.4 [31–33]. Although data on pure culture were not available in the literature, activa-

tion energies for INT reduction by both strains were within the above range.

The ratio of FFC to TC for both strains of SRB decreased with culture age and starvation time, the difference between total and viable counts indicated the number of inactive cells. It was noted that the differences between PC, FFC and TC increased with the starvation time. A similar phenomenon was also observed when starved marine aerobic bacteria were exposed to Cd^{2+} [34]. The proportions of FFC to TC for bacterial communities vary among various aquatic environments; e.g., 2–18% in freshwater [9,11], 0.5–4.0% in seawater [9,13], and 5–16% in water of saltmarsh [10].

The increase in the ratio of respiring, but not growing cells, to TC in starved SRB is particularly interesting from the ecological viewpoint of SRB in natural environments. This may be a key to resolve the problem presented by Jørgensen [35]. He showed that viable counts of SRB using a lactate medium underestimated the population number in coastal sediment by up to approx. 1000-fold, judged by comparison between the sulfate reduction rate per colony forming unit in the sediment and those in pure culture of SRB. Should be that many SRB in the sediment were able to respire, but unable to produce visible colonies on agar media, because FFC was 50–100 times higher than PC after 300 h of starvation in both strains (Fig. 5). Of course, other factors involving the supposition of Jørgensen [35] may have caused this discrepancy. It is necessary to count respiring SRB directly in natural environments, using the method combined with the INT technique and immunofluorescence.

ACKNOWLEDGEMENTS

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Effect of dilution on methanogenesis, hydrogen turnover and interspecies hydrogen transfer in anoxic paddy soil

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1. SUMMARY

Dilution of anoxic slurries of paddy soil resulted in a proportional decrease of the rates of total methanogenesis and the rate constants of H_2 turnover per gram soil. Dilution did not affect the fraction of H_2/CO_2-dependent methanogenesis which made up 22% of total CH_4 production. However, dilution resulted in a ten fold decrease of the H_2 steady state partial pressure from approximately 4 to 0.4 Pa indicating that H_2/CO_2-dependent methanogenesis was more or less independent of the H_2 pool. The rates of H_2 production calculated from the H_2 turnover rate constants and the H_2 steady state partial pressures accounted for only $< 5\%$ of H_2/CO_2-dependent methanogenesis in undiluted soil slurries and for even less after dilution. Upon dilution, the Gibbs free energy available for H_2/CO_2-dependent methanogenesis decreased from -28.4 to only -5.6 kJ per mol. The results indicate that methane was mainly produced from interspecies H_2 transfer within syntrophic bacterial associations and was not significantly affected by the outside H_2 pool.

2. INTRODUCTION

Dilution may have various effects on the microbial metabolic processes in anoxic methanogenic sediments or other environmental samples. Dilution results for example in a reduction of activity per unit size of sample, since the number of microorganisms is diluted. Dilution also results in a concentration change of metabolites and other substances which may be effective in regulation of microbial activities. Finally, dilution changes the spatial organisation of microorganisms and their metabolites within an environmental sample. In methanogenic environments dilution may have especially serious effects, since methanogenesis is accomplished by a complex microbial community. In this community H_2-producing fermenting and H_2-consuming methanogenic bacteria syntrophically depend on each other, since H_2 concentrations have to be regulated within narrow limits to allow for exergonic H_2 production as well as exergonic H_2 utilization [1,2].

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Because of its deleterious effects, dilution should be avoided as much as possible for investigating microbial processes in methanogenic samples. If sediment samples are rather liquid, this is easily accomplished, since substrates, radiochemical tracers etc. can rapidly be mixed into the sample. In stiff sediments, however, which have a relatively low content of water-filled pores, adding substrates or effectors can be difficult. In some cases, the core-injection technique may be applied [3]. In many other cases, however, the only choice will be to prepare a slurry.

For measuring H_2 turnover rates and H_2/CO_2-dependent methanogenesis in anoxic paddy soil it was necessary to introduce, equilibrate and extract H_2, $^{14}CO_2$ and $^{14}CH_4$ from the sample with a gaseous headspace. Since the water content of paddy soil is relatively low (15–30 g water per 100 g fresh weight) under field conditions, the experimental procedures required the preparation of slurries.

Therefore, we systematically studied the effect of dilution on H_2 turnover rate constants, H_2 steady state partial pressures, methane production rates and the fraction of H_2/CO_2-dependent methanogenesis in slurries of anoxic paddy soil. The results indicate that most of the H_2/CO_2-dependent methanogenesis takes place within syntrophic microbial associations which are not dependent on the outside H_2 steady state concentration for CH_4 production and thus, are relatively insensitive to dilution.

3. MATERIALS AND METHODS

3.1. Soil slurries

The soil was collected from paddy fields of the Italian Rice Research Institute in Vercelli. Storage and handling was done as previously described [4]. Slurries were prepared by adding 1 ml of water to each 1 g of dry weight soil giving a concentration of 0.75 g d.w. per ml slurry. The slurries were incubated under an atmosphere of N_2/CO_2 (8 : 2) at a temperature of 25 °C. After 3 days the colour of the soil had turned from brown to grey, and CH_4 production had started. Experiments were initiated 10 to 16 days after preparation of the slurries. Dilutions were done with O_2-free distilled water.

3.2. Methane production and H_2 steady state partial pressures

Serum bottles (120 ml) were filled with different amounts of diluted or undiluted anoxic soil slurries, closed with a butyl rubber stopper, flushed with N_2, pressurized to a total pressure of 150 kPa, and incubated at 25 °C. The following days, the headspace was repeatedly analyzed for H_2 and CH_4. The bottles were shaken vigorously for 30 s before gas samples were taken to ensure equilibration between liquid and gas phase. The pH of the anoxic soil slurry was determined at the end of the experiment. Gibbs free energies (ΔG) of H_2-dependent methanogenesis under the actual incubation conditions were calculated as described previously [2].

3.3. Hydrogen turnover rate

The anoxic soil slurry was anaerobically transferred into a glass syringe (200 ml) avoiding a gaseous headspace and incubated at 25 °C. H_2 was added as aqueous solution, and the decrease of dissolved H_2 was analyzed after extraction with H_2-free air as described previously [5,6]. The H_2 turnover rate constant (k_{H2}) was determined from the logarithmic decrease of H_2 at limiting H_2 concentrations (10 μM). In steady state, the H_2 production rate (P_{H2}) which is equivalent to the H_2 turnover rate was calculated from its turnover rate constant, the steady state H_2 partial pressure (p_{H2}), the Bunsen solubility coefficient (α) of H_2 and the molar volume (v) of an ideal gas at 25 °C:

$$P_{H2} = (\alpha/v) k_{H2} p_{H2}$$

3.4. Fraction of H_2 / CO_2-dependent methanogenesis

Serum bottles (57 ml) were filled with 20 ml of diluted or undiluted anoxic soil slurries, closed with butyl rubber stoppers, evacuated and gassed with N_2. The experiment was initiated by addition of 100 μl of carrier-free $NaH^{14}CO_3$ solution (6.6×10^6 dpm; 50 nmol). The bottles were incubated in duplicate at 25 °C. After 24 and 48 h, the headspace was analyzed for total CH_4, CO_2 and H_2 as well as for radioactive $^{14}CH_4$ and $^{14}CO_2$.

Total CH_4 and CO_2 (μmol ml^{-1} of headspace) as well as $^{14}CH_4$ and $^{14}CO_2$ (dpm ml^{-1} of headspace) increased linearly with time giving a constant specific radioactivity (dpm μmol^{-1}) of CH_4 and CO_2 over the incubation period of 48 h. The fraction of H_2/CO_2-dependent methanogenesis (f_{H2}) was calculated from the specific radioactivities of CH_4 (SR_{CH4}) and CO_2 (SR_{CO2}):

$$f_{H2} = SR_{CH4}/SR_{CO2}$$

3.5. *Gas analysis*

Analysis of H_2, CH_4 and CO_2 was done as described previously [4]. $^{14}CH_4$ and $^{14}CO_2$ were analyzed in a gas chromatograph at 50°C using a Porapak QS (80/100 mesh) separation column (length = 3 m; i.d. = 4 mm) and H_2 as carrier gas at a flow rate of 25 ml min^{-1}. Behind the separation column the gas stream was passed through a methanizator (Ni-Catalyst at 330°C; Chrompak, Middelburg, Netherlands). The gas stream was then split and fed into a flame ionisation detector (Carlo Erba, Milano, Italy) and a RAGA radioactivity detector for gas chromatography (Raytest, Straubenhardt, F.R.G.). The radioactivity signal was calibrated by using $^{14}CO_2$ which was prepared from $NaH^{14}CO_3$ standard solutions.

4. RESULTS

Incubation of anoxic slurries of paddy soil resulted in a linear increase of CH_4 in the headspace and in the establishment of a more or less constant H_2 partial pressure after 1–2 days of incubation [4]. Fig. 1 illustrates an experiment in which anoxic water was added to a soil slurry. The addition diluted the slurry and decreased the ratio of the volumes of gas headspace (V_g) to slurry (V_s) in the serum bottle. While the addition of water resulted only in a small decrease of CH_4 production, it dramatically reduced the H_2 steady state partial pressure from approximately 4 to 0.4 Pa.

Similar experiments with different amounts of water addition are summarized in Fig. 2. In these experiments the total amount of soil dry weight (7.5 g) was constant, but the concentration of the soil slurry changed between 0.75 g d.w. (con-

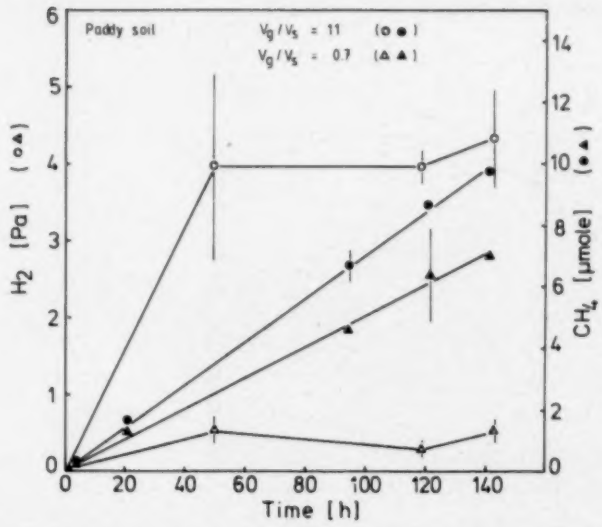

Fig. 1. Increase of CH_4 (closed symbols) and H_2 (open symbols) in undiluted and diluted slurries of anoxic paddy soil. Serum bottles (120 ml) contained 7.5 g d.w. soil as slurry which had been preincubated for 10 days under anoxic conditions at 25°C. The slurry was either undiluted (10 ml slurry; V_g/V_s = 11; circles) or diluted with anoxic water (70 ml slurry; V_g/V_s = 0.7; triangles). The data points are average values ± S.D. from triplicate experiments.

centration factor = 1.0) and 0.075 g d.w. (concentration factor = 0.1) per ml of soil slurry. Simultaneously the volume ratio (V_g/V_s) changed from 11 to 0.2. The H_2 partial pressure decreased by a factor of ten due to addition of water. The methane production rates, on the other hand, only decreased to 70% of the undiluted activity. This small decrease of CH_4 production may have been due to the decrease of the headspace volume rather than of the slurry concentration. For reduction of the headspace volume at a constant concentration of the slurry also decreased CH_4 production rates to 80%, while H_2 steady state partial pressures were only slightly reduced (data not shown).

In order to rigorously test whether the decrease of the H_2 steady state partial pressure was due to dilution of the slurry or due to the reduction of the volume of the gas headspace, the experiment was repeated by keeping the volume ratio constant at $V_g/V_s = 11$ and varying the concentration of the soil slurry between 0.75 g d.w. per ml (concentration factor = 1) and 0.075 g d.w. per ml (concentration factor = 0.1) (Fig. 3). Again, the H_2 steady state partial pressure was by a factor of 10 lower if the slurry was diluted to concentration factors < 0.6. CH_4 production rates, on the other

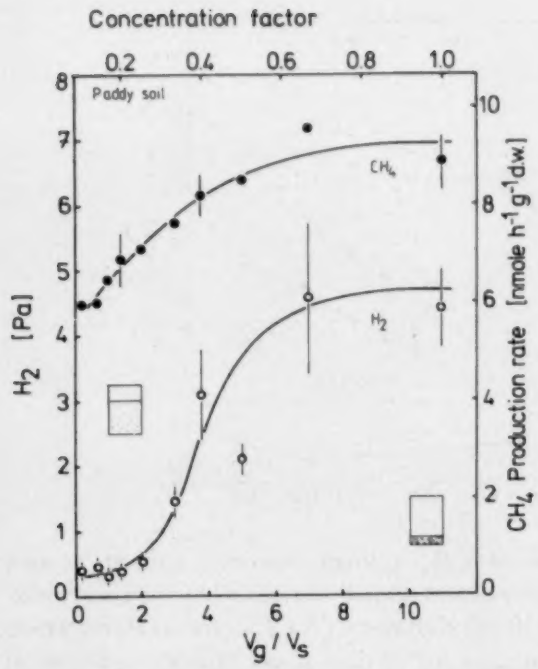

Fig. 2. CH_4 production and H_2 steady state partial pressure as function of headspace volume or concentration of anoxic soil slurry. The total amount of soil dry weight was kept constant, but the amount of water was varied. Serum bottles (120 ml) contained 7.5 g d.w. soil at increasing concentrations and increasing ratios of volumes (V_g/V_s) of headspace to slurry. The actual slurry concentration (gram d.w. soil per ml slurry) is calculated from the concentration factor $\times 0.75$. The slurry was preincubated anaerobically for 10 days, then the gas phase was replaced with N_2 and production of CH_4 and H_2 were measured as shown in Fig. 1. The rates of CH_4 production were calculated from the temporal increase of CH_4. The H_2 steady state partial pressures are average values $\pm$ S.D. from all data measured after 2 days of incubation.

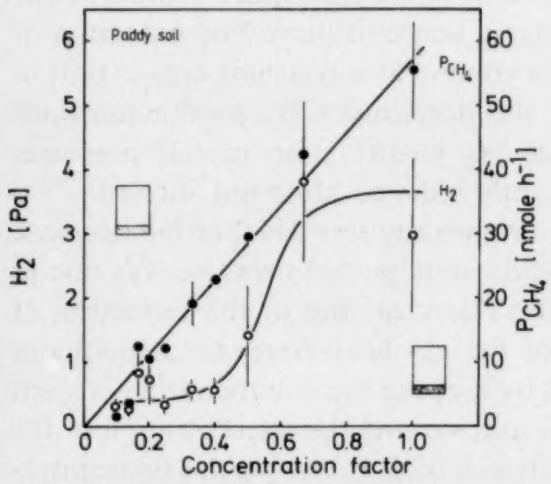

Fig. 3. CH_4 production and H_2 steady state partial pressure as function of the slurry concentration. The total amount of slurry and V_g/V_s was kept constant, but the concentration of the slurry was varied. Serum bottles (120 ml) contained 10 ml slurry at different concentration factors $\times 0.75$ g d.w. per ml; rest as in Fig. 2.

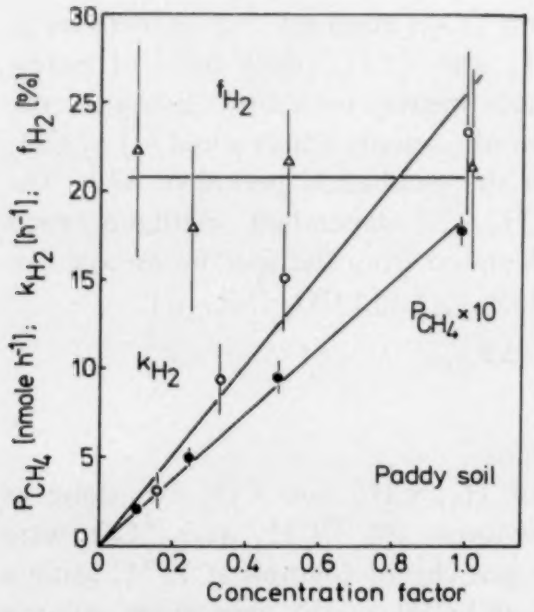

Fig. 4. H_2 turnover rate constant (k_{H2}), total CH_4 production rate (P_{Ch4}) and fraction of H_2/CO_2-dependent methanogenesis (f_{H2}) as function of slurry concentration. The actual slurry concentration (gram d.w. soil per ml slurry) is calculated from the concentration factor $\times 0.75$. The slurry was preincubated anaerobically at 25 °C for 16 days.

hand, were proportional to the concentration factor. These observations show that the H_2 partial pressure was affected by the concentration of the slurry rather than by the volume ratio of headspace to slurry.

Both, CH_4 production rate (P_{CH4}) and H_2 turnover rate constant (k_{H2}) were proportional to the concentration factor (Fig. 4) indicating that both parameters were controlled by the amount of soil, i.e. the amount of soil microorganisms, present in the test system. However, the fraction of CH_4 (f_{H2}) that was produced from H_2 and $^{14}CO_2$, was independent of the concentration factor and amounted to approx. 22%.

The data shown in Fig. 4 allow to calculate and compare the rates of H_2 production (equivalent to H_2 turnover), and H_2/CO_2-dependent CH_4 production. The results are shown in Fig. 5. The rates of H_2 production were more than 10 times smaller than necessary to explain the rates of H_2/CO_2-dependent methanogenesis which is based on a stoichiometry of 1 CH_4 produced from 4 H_2. Therefore, more than 94% of the methanogenic activity must be due to interspecies H_2 transfer (ISHT) between juxtaposed H_2-producing and methanogenic bacteria in order to explain the

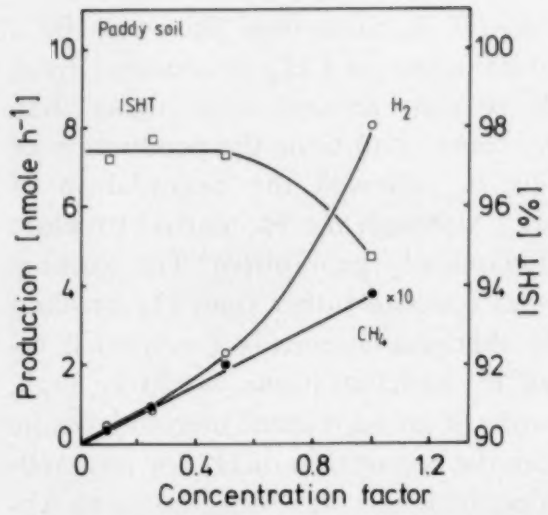

Fig. 5. Comparison of the H_2 production rates, of the rates of H_2/CO_2-dependent methanogenesis and of the percentage contribution of interspecies H_2 transfer (ISHT) to H_2/CO_2-dependent methanogenesis at different slurry concentrations (concentration factor × 0.75 g d.w. ml^{-1}).

observed rates of H_2/CO_2-dependent methanogenesis. In addition, while H_2/CO_2-dependent CH_4 production rates increased proportionally to the concentration factor, H_2 production rates increased in a nonlinear way. Therefore, the percentage contribution of interspecies H_2 transfer to H_2/CO_2-dependent methanogenesis was maximal

Table 1

Gibbs free energies of H_2-CO_2-dependent methanogenesis in undiluted and diluted slurries of anoxic paddy soil at 25 °C

Parameter	Soil slurry	
	undiluted (7.5 g d.w./ml)	diluted (0.075 g d.w./ml)
H_2 (Pa)	2.57	0.24
CH_4 (Pa)	509	36
HCO_3^- (mM)	4.2	0.45
pH	7.3	7.5
ΔG (kJ/CH_4)	−28.4	−5.6
CH_4 production from H_2/CO_2 (nmol h^{-1} g^{-1} d.w.)	2.54	2.01

The data were taken from the experiment described in Fig. 4 after 2 days of anaerobic incubation at 25 °C. H_2, CH_4 and pH were measured directly, bicarbonate was calculated from the CO_2 partial pressure and the pH. CH_4 production was calculated from the increase of $^{14}CO_2$-derived $^{14}CH_4$.

in dilute slurries while the contribution of the turnover of the H_2 pool was minimal.

The energetics of H_2/CO_2-dependent methanogenesis in concentrated and diluted soil slurries are compared in Table 1. In the diluted slurries the Gibbs free energies of CH_4 production were as low as −5.6 kJ per mol CH_4 produced (−1.2 kJ per mol H_2 utilized), about 5 times lower than in the concentrated slurries. Nevertheless, rates of H_2/CO_2-dependent methanogenesis were similar on a gram soil basis, indicating that methanogens were not limited by the energy available from the steady state H_2 partial pressure of the free H_2 pool.

5. DISCUSSION

In anoxic paddy soil, dilution of slurries did not significantly influence the rates per gram soil of H_2/CO_2-dependent methanogenesis, but strongly decreased the steady state concentrations of the H_2. The production rate of H_2 into the H_2 pool was less than 5% of the rate required to sustain the rate of H_2/CO_2-dependent methanogenesis and was even less in diluted than in concentrated slurries. The most conceivable explanation for this observation is the existence of syntrophic bacterial associations in which fermenting bacteria are juxtaposed to methanogenic bacteria and H_2 (or other reducing equivalents) are transferred directly without equilibrating completely with the outside pool of H_2. Similar observations have been made in a lake sediment [5,6] and in anaerobic sewage sludge [5,7]. Upon dilution, these syntrophic associations seem to stay intact and operative as long as the supply of fermentable substrates is not limiting which, however, was apparently not the case with concentration factors of up to 0.1.

The effect of dilution and the decrease of the H_2 partial pressure on the fermentation pattern of carbohydrates and the further degradation of the fermentation products is yet unknown. One might expect that the fermentation of carbohydrates is shifted towards more oxidized (e.g., acetate) versus reduced (e.g., ethanol, fatty acids) products when H_2 is decreased [8]. The enhanced production of

acetate may have stimulated acetate-dependent methanogenesis and thus, may explain why dilution apparently did not significantly influence the rates per gram soil of total methanogenesis. The degradation of the fermentation products (e.g., ethanol, lactate, fatty acids), on the other hand, should not have been significantly affected by dilution, if it took place inside the syntrophic methanogenic flocs which shield the fermenting bacteria from the outside H_2 partial pressures. The substrates of the syntrophic flocs are yet unknown, but glucose, lactate, and ethanol strongly stimulated production of $^{14}CH_4$ from $^{14}CO_2$ while propionate had no effect (Conrad and Babbel, in prep.).

The existence of interspecies H_2 transfer in syntrophic microbial associations is also consistent with thermodynamics of CH_4 production. In diluted slurries H_2 partial pressures were so low that H_2/CO_2-dependent methanogenesis would be only slightly exergonic if operating with this H_2 pool. The calculated Gibbs free energy change of -1.2 kJ per mol H_2 was significantly less than the values (-6.7 to -10.4 kJ per mol H_2) observed in diverse methanogenic environments under in-situ conditions [2] and was also significantly less than the lowest values (-6.5 kJ per mol H_2) observed in pure cultures of methanogenic bacteria [9,10]. In diluted slurries the Gibbs free energy available for H_2/CO_2-dependent CH_4 production (-4.9 kJ per mole of CH_4) would also be significantly less than the energy required for synthesis of 1/3 ATP (about -14.7 kJ) which is presently assumed to be the minimum size of biological energy quantum [11]. However, within syntrophic microbial association H_2 may be transferred directly and thus, the thermodynamic situation for methanogens may be more favourable than assumed from the outside H_2 pool. In fact, it is possible that the bulk of the outside pool of H_2 is the result of leakage of H_2 from the syntrophic associations rather than of H_2 production by free, unassociated fermenting bacteria.

Thiele and Zeikus [12] recently presented experiments which indicate that in anaerobic digestor flocs methanogenesis may be mediated via interspecies formate transfer rather than interspecies H_2 transfer. Formate was shown to be a significant intermediate in CH_4 production from ethanol, if H_2 partial pressures were higher than 10 kPa. Under these conditions the production of formate versus H_2 allowed the degradation of ethanol to CH_4, although the H_2 partial pressure was thermodynamically prohibitive. The authors pointed out that formate rather than H_2 production would be thermodynamically favoured if bicarbonate and H_2 concentrations would be high, basically because of an additional thermodynamic advantage from the conversion of H_2 (or intracellular reducing equivalents) plus bicarbonate to formate: $H_2 + HCO_3^- \rightarrow HCO_2^- + H_2O$ ($\Delta G^{\circ\prime} = -1.3$ kJ).

In anoxic paddy soil, however, formate production would be unfavourable because of the low H_2 partial pressures. The data of H_2 partial pressures and bicarbonate concentrations measured in diluted and undiluted soil slurries (Table 1) allow to calculate the formate concentration at which formate production from H_2 and bicarbonate would be exergonic. The permissive formate concentration would be in a range of < 180 nM for undiluted and < 1.8 nM for diluted soil slurries. However, analyzed formate concentrations were in a range of 10 to 70 μM (unpublished results) so that formate production would be thermodynamically less favourable than H_2 production. In addition, interspecies formate transfer is unlikely in anoxic paddy soil, since the prevalent methanogens seem to be of the *Methanobacterium bryantii* type (Seitz, Mayer and Conrad, in prep.) which is unable to utilize formate. In the anoxic digestor flocs, by contrast, the prevalent methanogen is the formate-utilizing *M. formicicum* [12,13]. Therefore, we conclude that in anoxic paddy soil methane is produced by interspecies H_2 transfer rather than by interspecies formate transfer.

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Photosynthesis and respiration of a diatom biofilm cultured in a new gradient growth chamber

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1. SUMMARY

A diatom biofilm was grown in a chamber developed for culture of biofilms in chemical gradients. The diatoms grew on a polycarbonate membrane filter which separated a sterile reservoir, with added phosphate, from a reservoir without phosphate. Within 3 weeks of inoculation, a thick biofilm developed on the surface of the filter. The biofilms were homogeneous and therefore suitable for calculations of O_2 diffusion fluxes from concentration profiles of O_2. Profiles of O_2, pH, and gross photosynthesis at different light intensities and liquid medium concentrations of dissolved inorganic carbon and O_2 were measured with microelectrodes. Respiratory activity in a layer of the biofilm was determined as the difference between gross photosynthesis and outflux of O_2 from that layer. The photosynthetic activity in a well-developed biofilm grown at 360 μEinst m^{-2} s^{-1} and 2.4 mM HCO_3^- was limited by the supply of inorganic carbon. Exposure to light above 360 μEinst m^{-2} s^{-1} stimulated gross photosynthesis as well as respiratory processes without affecting net outflux of O_2. Higher concentrations of inorganic carbon, on the other hand, enhanced gross photosynthesis without concurrent increase in respiratory rate, resulting in an increased outflux of O_2. High concentrations of O_2 in the liquid medium decreased the net outflux of O_2 with little effect on the gross photosynthesis. The effects of inorganic carbon and O_2 on the metabolic activities of the biofilm were consistent with the presence of photorespiratory activity.

2. INTRODUCTION

Growth and photosynthetic capacity of biofilms in aquatic environments have been described in the past [1–3]. However, the accurate measurement of photosynthetic and respiratory processes was hampered by the high rates of metabolism within thin layers [3,4]. It was necessary to examine the biofilm as an entity of autotrophic and heterotrophic organisms and to measure the combined activity from either the net O_2 flux to the surroundings or from the uptake of radiolabeled CO_2. There are methodological problems by these 'black box' approaches for determinations of photosynthetic activities [4,5] and they do not yield information about the concurrent respiratory processes which also may be stimulated during

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illumination. The traditional methods are also insufficient to detect the stratification of metabolic activities within the biofilms. These analytical difficulties have led to attempts to simulate the growth conditions by computer models and then deduce rates of respiration and photosynthesis from these simulations [6]. The computer simulations inferred that diffusion limitations imposed on the biofilm by the diffusive boundary layer in the water just above the biofilm and by the biofilm itself could result in chemical conditions (high O_2, low CO_2) within the biofilm favoring photorespiration and limiting the growth and thickness of the biofilm.

The appearance of microelectrode based methods to quantify photosynthesis [4,7] and respiration [8] now allow us to study in much more detail the metabolic processes within biofilms and similar substrates. The stratification of gross photosynthesis in sediments, algal mats, and epiphytic biofilms have been described in detail [9]. Depth profiles of respiratory activity in an algal mat have also been calculated [8]. However, experimental data on the variation of respiratory activity with changing environmental conditions have never been published, and such data are important for the interpretation of gross photosynthesis data obtained by use of O_2 microelectrodes. Our purpose was to describe photosynthetic and respiratory activities of a cultured diatom biofilm as a function of light intensity and of O_2 and inorganic carbon concentrations in the overlying water. A growth chamber was developed for culture of homogeneous biofilms especially suited for such investigations where it is essential that measurements for gross photosynthesis and O_2 diffusion gradients can be performed with high accuracy and reproducibility. The biofilms grew in a gradient of dissolved orthophosphate which restricted growth to the permeable surface of a filter disc.

3. MATERIALS AND METHODS

3.1. Culture of biofilm

The diatom biofilm was cultured in a growth chamber (Fig. 1) which was developed especially to maintain a chemical gradient through the biofilm. The autoclavable chamber was made of 3-mm thick polycarbonate sheets sealed together with silicone rubber and consisted of two enclosures, A (100 ml) and B (200 ml) separated by a 0.22 μm Nuclepore membrane filter of 1.2 cm in diameter. The filter was sealed to the polycarbonate wall with silicone rubber. Enclosure A was kept sterile and contained an inorganic nutrient medium containing (in g/l): NaCl 10; $NaNO_3$ 0.75; K_2HPO_4, $3H_2O$ 0.01; Na_2CO_3 0.01; EDTA 0.005; $FeCl_2$, 6 H_2O 0.0031; and citric acid 0.0054. Enclosure B contained a mineral medium without added phosphate containing (in g/l): NaCl 9.5; $MgSO_4$, $7H_2O$ 1.2; $MgCl_2$, $7H_2O$ 0.8; KCl 0.2; $CaCl_2$, $2H_2O$ 0.37, $NaHCO_3$ 0.2; KBr 0.07; $SrCl_2$, $6H_2O$ 0.015; NaF 0.002; Na_2SiO_3, $9H_2O$ 0.001. To enclosure B vitamins were added in the following final concentrations: B_1 0.5 mg/l; B_{12} 1 μg/l; biotin 0.5 μg/l, and 1 ml/l of a trace metals solution [10]. The media both had a salinity of 12‰ but the pH was 7.8 in A and 8.1 in B. New media were continuously pumped through the chambers at rates of 1 ml h^{-1} in A and 10 ml h^{-1} in B. Because of the continuous renewal of media, sufficient nutrients to support high growth rates of photosynthetic organisms were present only at the membrane surface in enclosure B. The silicone rubber seal (not shown in Fig. 1) between the lid and the rest of the chamber could be cut and the lid removed to enable analysis by microelectrodes. The polycarbonate walls allowed illumination of the B side of the filter surface with little changes in the spectral composition of the light. A halogen lamp was used as a light source, and a 7 mm glass sheet was inserted as an infrared filter between the lamp and the chamber. The biofilms were cultured at a light intensity of 360 μEinst m^{-2} s^{-1} which, in spite of the infrared filter, resulted in an increase of the temperature in the growth chamber to 25°C which was 3°C above ambient temperature. A slide projector with a halogen lamp and equipped with neutral density filters to reduce the light intensity was used in experiments where the response of the biofilm to various light intensities was investigated. The infrared filter in the slide projector was very efficient so that the temperature elevation in the growth chamber was insignificant.

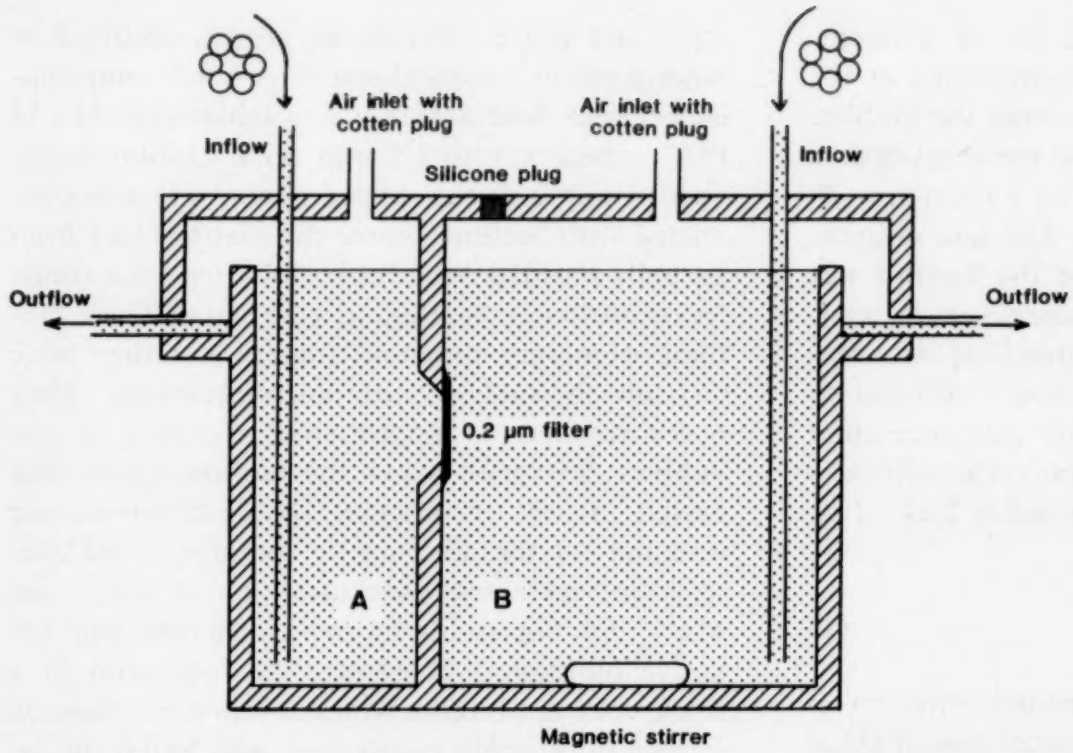

Fig. 1. Growth chamber for culture of microbial biofilms in concentration gradients of dissolved chemical species. In the present study, the medium pumped through the sterile compartment A contained dissolved phosphate whereas the medium pumped through compartment B contained no phosphate. The almost complete absence of phosphate in the bulk of compartment B limited growth of a diatom biofilm to the surface of membrane filter separating the two compartments. The chamber had the following dimensions: Length 12 cm, width 4.5 cm, height 8 cm.

The diatom used, *Amphora* sp., was isolated in unialgal culture from a shallow estuarine sediment. The enrichment was performed in the growth chamber to ensure that the isolated diatom would grow under these conditions. After 3 weeks a second growth chamber was inoculated from the first one, and after an additional 3 weeks an almost monoalgal biofilm had developed. An inoculum from this biofilm was streaked onto an agar supplemented with inorganic nutrients [11]. Addition of antibiotics to eliminate bacteria was not performed. Diatoms from one of the small colonies which developed after 10 days incubation under a fluorescent lamp were transferred to a new agar plate, and the unialgal culture (verified by microscopic examination) was hereafter maintained on agar. Inocula from the plates were homogenized for a few seconds in a few ml of mineral medium using an Ultra Turrax homogenizer (Janke and Kunkel KG, D-7813 Staufen, F.R.G.) before injection through the silicone injection port in the lid of the growth chamber. Contamination of the inocula with new bacteria or eucaryotic grazers was avoided during this transfer using sterile homogenizer head, syringes, etc.

3.2. Microelectrode analysis

O_2 and gross photosynthesis were determined by use of O_2 microelectrodes [9,12]. The applied O_2 microelectrodes all exhibited a difference in signal between stirred and stagnant water of less than 4%, and a correction for this artefact was not necessary. A recent publication [13] suggests that data obtained by use of oxygen microelectrodes are inaccurate, but the observed inaccuracy in their experiments was due to poor microelectrodes exhibiting a 20–50% change in signal by stirring. Profiles of pH in the biofilm were determined by use of pH microelectrodes [9]. The flow through the enclosures was stopped during the recording of microelectrode data, and the lid of the chamber was removed. Continuous stirring and gentle aeration were performed by bubbling air through the medium from a bundle of 10 glass capillaries each having an inner diameter of approx. 0.05 mm. The microelectrodes were inserted in a 46° angle from

horizontal into the biofilm by use of a micromanipulator which allowed measurements at 0.1 to 0.025 mm depth intervals through the biofilm. The O_2 concentrations as well as the exact depths were automatically transferred to a computer [9] and stored on magnetic media. The rate of gross photosynthesis in each layer of the biofilm was calculated from the rate of decrease in O_2 concentration 1 second after darkening [7,8]. An accurate manual recording of this rate is difficult to perform, but the computer did the calculation with a very good reproducibility. The diffusion flux (J) of O_2 was calculated by use of Fick's first law of diffusion [9]:

$$J = -D_s \frac{\delta C(x)}{\delta x} \quad (1)$$

where D_s is the apparent diffusion coefficient in the biofilm and $C(x)$ is the concentration of O_2 at depth x. D_s was calculated from a steady state O_2 profile in an inactivated biofilm which was placed in a steep O_2 gradient. The 'diffusion coefficient' determined in this way also included a correction factor for a decrease in porosity relative to pure water. The determination of diffusion coefficients by use of O_2 microelectrodes will be published in detail elsewhere (N.P. Revsbech, unpublished data). The diffusion coefficient in the biofilm at 22°C, at which temperature all measurements were done, was determined to be 1.1 $cm^2\ s^{-1}$. The corresponding value for water at 22°C [14] and 12‰ salinity [15] is 2.1 $cm^2\ s^{-1}$. The rate of respiration by the biofilm was calculated as the difference between the integrated rate of gross photosynthesis as measured by the microelectrode technique and the net outflux of O_2 (flux to the overlying water plus downwards towards the filter). The activities within a layer of the biofilm could be calculated by integrating over a depth interval and calculating the fluxes at both boundaries.

4. RESULTS

4.1. Culture of biofilm

Nutrient media with various concentrations of phosphate were tested to determine a concentration where growth on the filter was relatively rapid and where virtually no growth occurred in other parts of compartment B. A good compromise between these demands was achieved at 44 μM PO_4^-, where a 1 to 1.5 mm thick biofilm developed within 3 weeks. Experiments were also conducted with biofilms where the diatoms had been partially purified from bacteria by repeated transfer on antibiotic-containing agar plates. These biofilms were very thin and fragile, and they were difficult to analyze with microelectrodes. They never reached an appreciable thickness as gas bubbles developed below the biofilm which then peeled off the membrane. Divalent cations are essential for the adhesion of diatoms to surfaces [16], but increased concentrations of Ca^{2+} and Mg^{2+} did not result in more coherent and adhesive biofilms. Apparently, the formation of a thick, coherent biofilm with our strain of *Amphora* as the autotrophic component was based on extracellular polymeric material [17] excreted by bacterial species not present in the thin biofilm.

Fig. 2 shows O_2 and photosynthesis profiles within a well developed biofilm with a diverse bacterial flora, and a thin biofilm with few species of bacteria, respectively. The photosynthetic activity per unit volume was about 5 times higher in the thin biofilm, but the photosynthetic rates integrated over all layers were approximately identical for the two biofilms.

4.2. Microelectrode analysis

The photosynthetic and respiratory activity of the biofilm was determined as a function of: 1) light intensity at 2.14 mM HCO_3^-, 2) light intensity at 0.12 mM HCO_3^-, 3) inorganic carbon concentration, 4) O_2 concentration. The experiments were designed to elucidate how the gross photosynthetic rates as measured by the O_2 microelectrodes were affected by the photorespiratory activity in the biofilm. Photorespiration is due to the oxygenase activity of the bifunctional enzyme ribulose 1,5 bisphosphate (RUBP) carboxylase/oxygenase [18] and has no energy yielding function. Photorespiration stops shortly after light is extinguished [19] due to diminishing substrate concentrations, but by the microelectrode technique the rate of photosynthesis is determined only 1 s after the darkening.

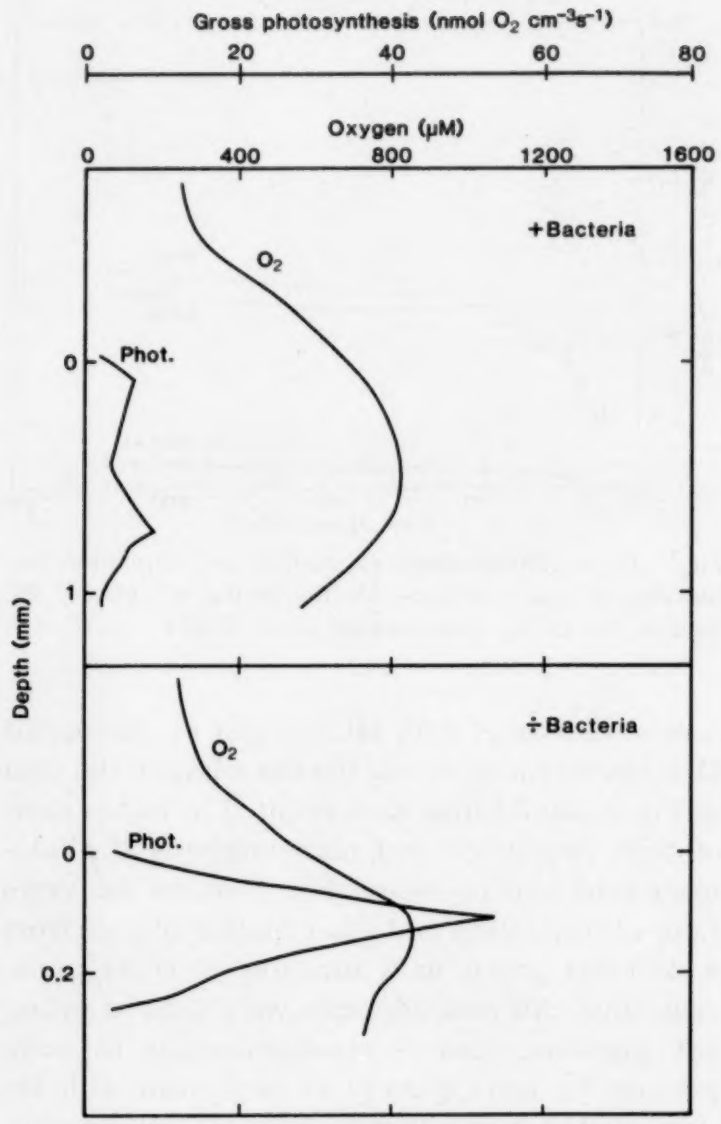

Fig. 2. Profiles of O_2 and gross photosynthesis in a biofilm with a diverse bacterial flora and in a poorly developed biofilm with a restricted bacterial flora. Note different depth scales. Light intensity 200 μEinst m^{-2} s^{-1}.

4.3. Effect of light intensity at 2.4 mM HCO_3^-

A series of experiments were conducted to observe the effect of light intensity on gross photosynthesis and respiration in the biofilm. Profiles were measured when a new steady state was reached after a minimum of 30 min under the new light regime. Fig. 3 shows profiles of O_2 and gross photosynthesis measured in the same position of the biofilm at 5 different light intensities. The data from two other positions were very similar. The gross photosynthetic activity was integrated over all layers and the mean values from the three parallel experiments are plotted in Fig. 4. Although the biofilm was more homogeneous than

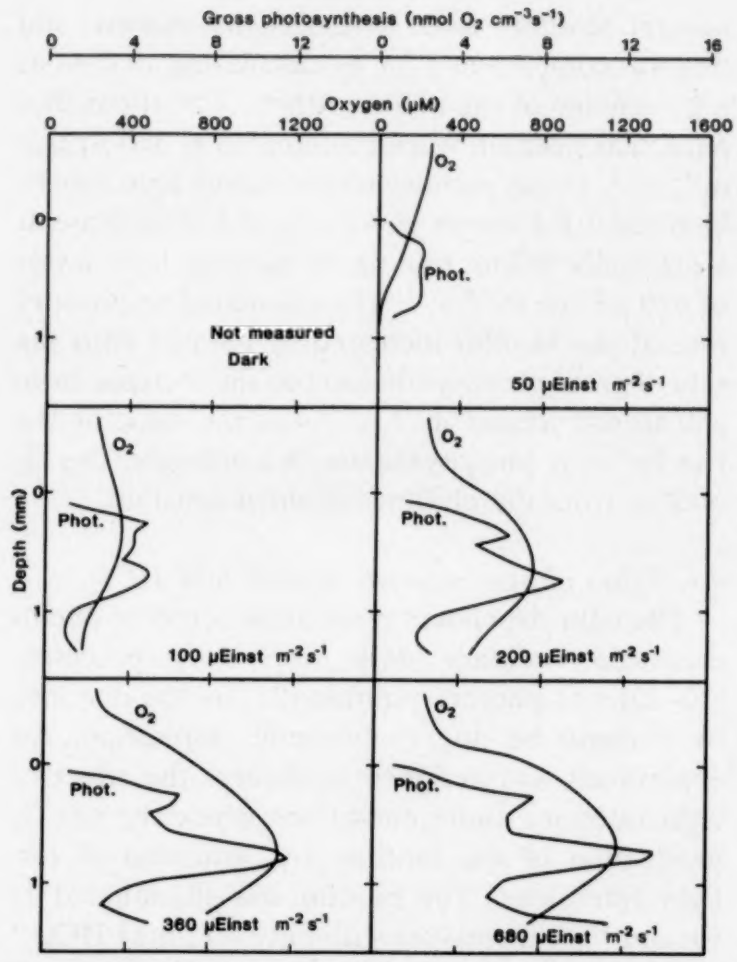

Fig. 3. Profiles of O_2 and gross photosynthesis at various light intensities and a HCO_3^- concentration of 2.4 mM.

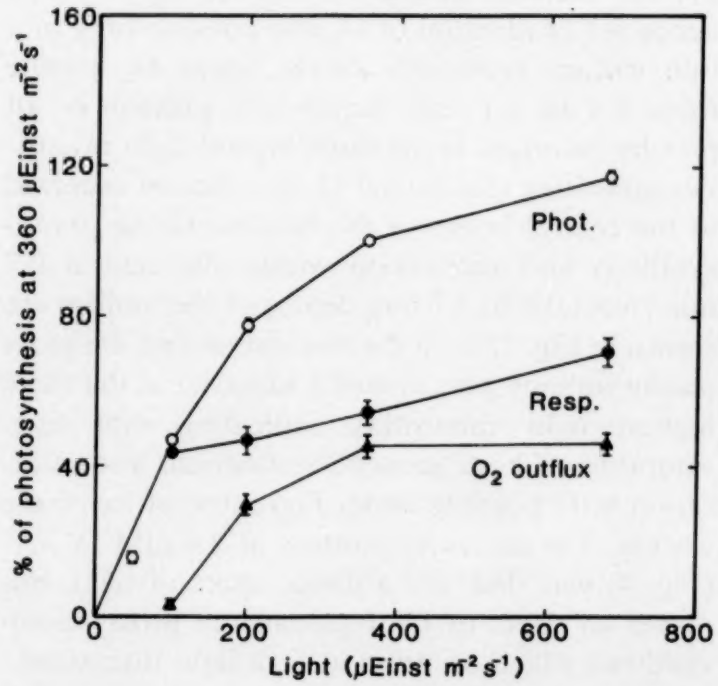

Fig. 4. Gross photosynthesis, O_2 outflux, and respiration as a function of light. The activities were calculated from data shown in Fig. 3 and 2 similar sets of data. Bars indicate standard error of the mean.

natural biofilms some heterogeneity existed, and this was compensated for by calculating all data as a percentage of the photosynthetic activity at that particular position when illuminated at 360 μEinst m^{-2} s^{-1}. Gross photosynthesis versus light resembled usual P-I curves and continued to increase at a gradually falling rate up to incident light levels of 680 μEinst m^{-2} s^{-1}. The calculated respiratory rate of the biofilm increased in parallel with the rate of gross photosynthesis, and the increase from 360 to 680 μEinst m^{-2} s^{-1} was the same as the rise in gross photosynthesis. Accordingly, the O_2 outflux from the biofilm remained constant.

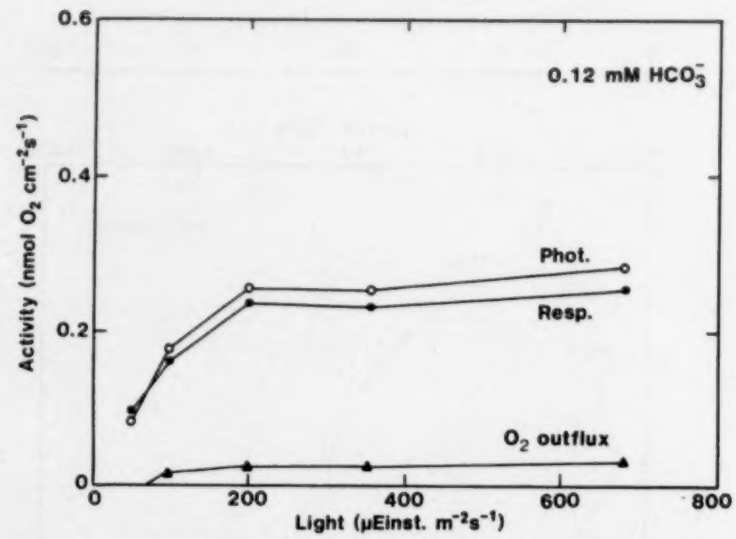

Fig. 5. Gross photosynthesis. O_2 outflux, and respiration as a function of light calculated for the central 0.7 mm of the biofilm. The HCO_3^- concentration was 0.12 mM.

4.4. Effect of light intensity at 0.12 mM HCO_3^-

The light-dependent respiration could be due to enhanced ordinary dark respiratory processes [20–22] and photorespiration [23] by the diatoms, or it could be due to bacterial respiration. An experiment was designed to observe the effect of light intensity under conditions where the net O_2 production of the biofilm was saturated at low light intensities. The biofilm was illuminated at various light intensities with only 0.12 mM HCO_3^- in the water. At this low concentration, all the HCO_3^- diffusing into the biofilm was consumed at the very surface, and a net fixation of CO_2 and hence net production of O_2 was possible only in a thin surface layer. An almost linear O_2 profile from 0.4 to 1.1 mm depth was present in all profiles recorded at the three highest light intensities indicating that no net O_2 production occurred in the central layers of the biofilm. Gross photosynthesis and respiration within the central 0.7 mm (from 0.4 to 1.1 mm depth) of the biofilm are shown in Fig. 5. Both the respiration and the gross photosynthesis were virtually identical at the three highest light intensities, indicating that light saturation of both gross photosynthesis and respiration were possible under limitation of inorganic carbon. The rise in respiration at 2.4 mM HCO_3^- (Fig. 4) was thus not a direct effect of light, but rather an effect of the high rates of gross photosynthesis which occurred at high light intensities.

4.5. Effect of inorganic carbon concentrations

Because of the bifunctionality of RUBP-carboxylase, photorespiration is affected by the concentrations of both HCO_3^- and O_2. Increased O_2 concentrations during the recording of the data in Fig. 5 should thus have resulted in higher rates of both respiration and photosynthesis if photorespiration was operative. Fig. 6 shows the mean rates of respiration and gross photosynthesis from 4 different points as a function of HCO_3^- concentration. All measurements were done at different positions, and a standardization to compensate for heterogeneity as performed with the data plotted in Fig. 4 was therefore not possible. The lack of a standardization resulted in relatively large standard errors. The respiration was stable at about 0.4 nmol cm^{-2} s^{-1} at all HCO_3^- con-

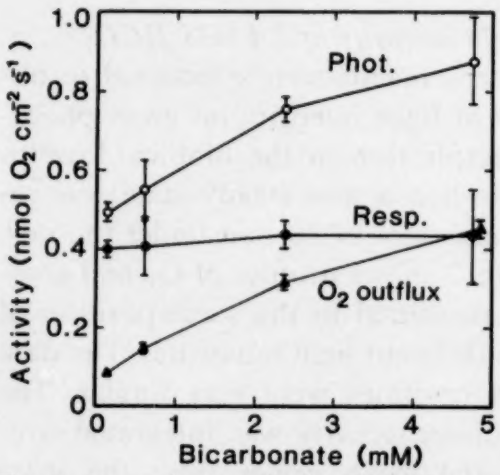

Fig. 6. Gross photosynthesis, O_2 outflux, and respiration as a function of HCO_3^- concentration at a light intensity of 360 μEinst m^{-2} s^{-1}.

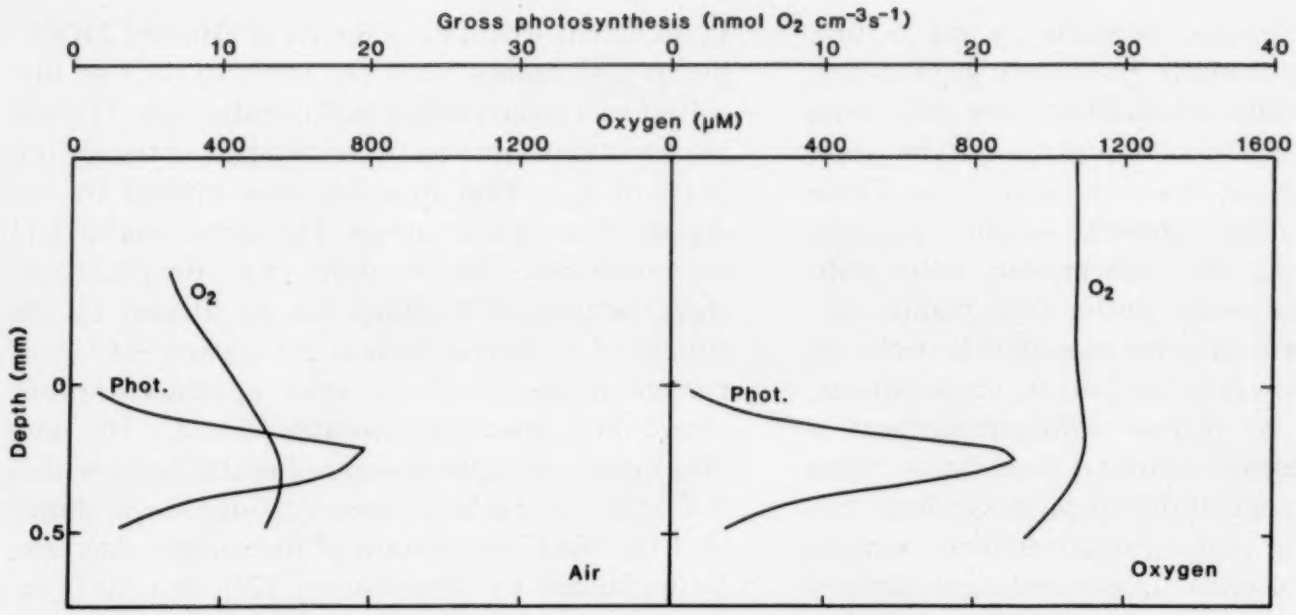

Fig. 7. Profiles of O_2 and gross photosynthesis during saturation of the waterphase with air (left) and with O_2 (right). Light intensity 360 µEinst m^{-2} s^{-1}. HCO_3^- concentration 2.4 mM.

centrations whereas the gross photosynthesis increased very significantly. Increased concentrations of inorganic carbon thus seemed to counteract the increasing respiratory rates with increasing photosynthetic rates and oxygen concentrations which were observed in Fig. 4. This could suggest that at least some of the increase in respiration seen in Fig. 4 was due to photorespiration.

Profiles of pH (not shown) recorded at 0.12 and 4.80 mM HCO_3^- exhibited maximum pH values of 9.57 and 9.85, respectively, in the inner parts of the biofilms. The concentration of available inorganic carbon, presumably HCO_3^- [24], inside the film was consequently limiting the rate of photosynthesis at an equilibrium pH of 9.57 at the low HCO_3^- concentration whereas this same limiting concentration was first reached at higher pH values at the higher alkalinities created by the higher HCO_3^- concentrations in the waterphase. The HCO_3^- concentration of 0.12 mM was somewhat underestimated as the pH 8.2 medium must have trapped some CO_2 from the air during the approx. 20 min of preincubation. The essential point, however, is that a wide range of inorganic carbon concentrations was tested.

4.6. Effect of O_2 concentration

Experiments with elevated O_2 concentrations in the overlying water were also performed. Increased O_2 concentrations should increase the rate of photorespiration [21,25] and thereby increase the difference between the gross rate of photosynthesis and the flux of O_2 out of the photosynthetic layer. Four experiments all showed such an effect, indicating that a significant fraction of the total respiration was due to photorespiration. Data from one of the experiments are shown in Fig. 7. The gross photosynthesis was 0.44 nmol cm^{-2} s^{-1} in air and 0.48 nmol cm^{-2} s^{-1} in O_2 saturated water. The corresponding figures for O_2 outflux from the biofilm were 0.17 and 0.12 nmol cm^{-2} s^{-1}, and the respiratory activities thus 0.27 and 0.36 nmol cm^{-2} s^{-1}, respectively.

5. DISCUSSION

5.1. Growth chamber

The growth chamber used in this study was well suited for culture of diatom biofilms. The nutrient flux through the filter created good conditions for growth of sessile microorganisms on the filter surface, whereas growth in suspension was prevented by low nutrient concentrations and high dilution rates. Just after inoculation, the increase in biomass on the filter must have been limited by phosphate and possibly light. In this paper we have shown that dissolved inorganic

carbon was a limiting substrate in the mature biofilm. Most previously published growth systems [26] for culture of biofilms have only been based on high dilution rates, and the biofilms have been growing on an inactive substratum. These previously published growth systems simulate growth conditions on impermeable, solid substrates in streams, water purification plants, etc., whereas the growth chamber described here closely simulates the growth conditions at the sediment-water interface. At shallow sediment surfaces, a high flux of inorganic nutrients from below often supports dense populations of photosynthetic microorganisms. The photosynthetic activity of natural populations of microalgae at sediment surfaces has been studied in detail using O_2 microelectrodes [9], but it has been difficult to determine rates of respiration from the microelectrode data due to the relatively great physical heterogeneity in natural communities. The more homogeneous biofilms cultured in the new gradient growth chamber made it possible to obtain rates of both photosynthesis and respiration during illumination.

5.2. Interpretation of data

There are three aspects associated with the respiratory activity in illuminated biofilms which shall be discussed here; 1) the physiological aspects, 2) the possible excretion of dissolved organic matter by photorespiring microorganisms, and 3) the relevance for the interpretation of photosynthetic rates obtained by use of O_2 microelectrodes.

5.2.1. Ecophysiology of microalgae

Many microalgae and cyanobacteria seem to be highly adapted to live under environmental conditions that should promote photorespiration, i.e., environments with high illumination, low concentrations of dissolved inorganic carbon, and high O_2 concentrations. The most important adaptation to avoid excessive photorespiration in such environments is probably the ability to actively concentrate the internal pool of dissolved inorganic carbon relative to the external pool [27,28]. However, in photosynthetically active biofilms, the inner parts of the biofilm may be deprived of HCO_3^- to an extent where even the most efficient HCO_3^- pump [24] cannot keep the internal pool of dissolved inorganic carbon sufficiently high. The net photosynthetic rate by the microalgae in the central parts of a biofilm thus becomes limited by the supply of inorganic carbon. The experimental data presented here clearly show that the photosynthetic activity of biofilms can be limited by the supply of inorganic carbon even when HCO_3^- is present in the overlying water at concentrations above those found in seawater (2 mM). The data also suggest that photorespiration did occur within the analyzed biofilm. Investigations have shown that the 'dark' respiration of microalgae may also be enhanced by illumination [29], but such respiratory activity should not be enhanced by low inorganic carbon and high O_2.

5.2.2. Excretion of organic compounds

The oxygenase action of RUBP carboxylase may have an indirect effect on the microbial community of the biofilm as glycolate, which is a product of the photorespiratory pathway, may be excreted in large amounts [30,31]. Some of the increase in respiratory activity observed under conditions promoting photorespiration may thus be due to bacterial degradation of these exudates. Coupling between excretion of photosynthetic exudates and growth of heterotrophic bacteria has previously been described in biofilms [2,32].

5.2.3. Interpretation of photosynthesis measurements

To most investigators measuring photosynthesis in natural environments, the primary productivity of the ecosystem is the central question. The primary production is usually defined as the production of new biomass per unit time. Such a definition is convenient in pelagic environments where most of the increase in biomass is consumed by higher trophic levels or sediments out of the system. In benthic photosynthetic communities where photosynthesis and respiration are generally more closely coupled, it is difficult to define a primary productivity. In this paper we have used two measures of photosynthesis to characterize the activity within such a community. One is the O_2 outflux and the other is the gross photosynthetic activity as measured by the O_2 microelectrodes.

The outflux of O_2 from the biofilm is equivalent to the net formation of biomass during illumination. By an axenic algal biofilm, the outflux would equal the net photosynthetic activity. There will only be an outflux of O_2 from a biofilm when the gross photosynthesis exceeds the respiratory processes. A biofilm with a high photosynthetic activity may have an influx of O_2 if the respiratory activity is even higher (data at 50 μEinst m^{-2} s^{-1} in Figs. 3 and 5), so the outflux of O_2 alone is not a satisfactory measure of photosynthetic activity.

Gross photosynthesis of oxygenic phototrophs can be defined as the O_2 evolved by the action of photosystem II. The O_2 evolution is proportional to the fixation of CO_2 in the light. In this paper we have used the term 'gross photosynthesis' for the rate of decrease in O_2 concentration one second after darkening as measured by the O_2 microelectrodes. Our determination of gross photosynthesis is only consistent with the definition given above if the rate of O_2 consumption by all processes remains constant within that one second, but that is generally the case [7]. The O_2 consumed almost immediately by photorespiration thus seems to be included in the gross photosynthetic rate as measured by the O_2 microelectrodes, and the determinations performed by use of O_2 microelectrodes give true gross photosynthetic rates. Such gross photosynthetic rates including O_2 consumed by a futile cycle only operating in the light are, however, of questionable ecological relevance if this cycle operates at an appreciable speed, and the results presented here do indicate that photorespiration may account for a significant fraction of the metabolism in illuminated biofilms.

The results presented in this paper do stress that convenient definitions [5] of terms as 'primary production' and 'photosynthetic activity' in microbial communities with closely coupled photosynthetic and respiratory processes are not easily found. We did define a gross photosynthetic rate which could be measured at high accuracy, but the ecological relevance of this parameter is difficult to evaluate. Combined measurement of both gross photosynthesis and O_2 outflux (i.e., net increase in biomass) as demonstrated in this paper may result in the best possible description of metabolic processes in photosynthetic biofilms and similar substrates.

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Validation of signature polarlipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials

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1. SUMMARY

Extractable cell membrane-derived polarlipid ester-linked fatty acids (PLFA) obtained from aerated soils gassed with methane or propane and from methane- and propane-oxidizing bacteria isolated from the soils were analyzed by capillary gas chromatography/mass spectrometry. Exposure of aerated soils to methane resulted in the formation of a high proportion of an unusual 18-carbon mono-unsaturated PLFA, 18 : lw8c. High proportions of this fatty acid biomarker are found in monocultures from this soil grown in minimal media with methane. This PLFA has been previously established as associated with authentic type II methane-oxidizing bacteria. The microbiota in aerated soils exposed to hydrocarbons containing propane, formed a suite of PLFA characterized by high proportions of a 16-carbon mono-unsaturated acid, 16 : lw6c, and an 18-carbon saturated fatty acid with an additional methyl branch at the 10 position, 10 Me 18 : 0. This PLFA pattern has been detected in several monocultures enriched from the soil with propane-amended minimal media. The correspondence of high proportions of these unusual mono-unsaturated PLFA in the isolated monocultures and in situ in the soils after stimulation with the appropriate hydrocarbon is a strong validation of the utility of these biomarkers in defining the community structure of the surface soil microbial community.

2. INTRODUCTION

The quantitative definition of the biomass and community structure of the microbiota of surface soils is one of the most pressing challenges of

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microbial ecology. Polarlipids, intercellular adenosine nucleotides, and cell wall amino-sugars are biochemical components of cells that have been utilized to estimate microbial biomass [1]. Smith et al. [2] have shown that there appears to be a unique microbial community in uncontaminated subsurface materials from below the root zone with a sparsely distributed microbiota which have an identical cocco-bacillary morphology. In these subsurface sediments the biomass and cell numbers estimated from direct cell count after acridine orange staining agree with the numbers and biomass estimated from the extractable polarlipid phosphate and total fatty acids, the total adenosine triphosphate, the fatty acids from the lipopolysaccharide lipid A, and the cell wall muramic acid content [3].

The ester-linked fatty acids in the polarlipids (PLFA) are presently one of the more sensitive and useful chemical measures of microbial biomass and community structure [4–6]. The use of fatty acids that are specifically ester-linked in the polarlipid fraction of the total lipid extract greatly increases the selectivity of this assay as many of the anthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. For example, by isolating the polarlipid fraction for fatty acid analysis it has proved possible to detect specific bacteria in the sludge of crude oil tanks (unpublished data).

Validations of the use of PLFA in defining microbial community structure have been reviewed [7]. These validations involved: modifying the microenvironment to induce changes that can often be predicted (changing nutrients or electron acceptors, adding antibiotics etc.), shifting the community nutritional status and observing the effects on formation of polymers and endogenous storage components, observing the effects on biofilms by grazers with known specificity, and isolating specific monocultures for PLFA biomarker analysis and showing the appropriate response in situ when that specific group of organism is induced or repressed.

In this study, PLFA biomarker techniques were applied to aerated subsurface materials or surface mucks (soils with high organic content) which were gassed with methane or propane containing hydrocarbons. The gassing induced the formation of microbial consortia with significantly increased proportions of specific PLFA. These specific PLFA were found to be characteristic of monocultures recovered from these experimental systems by selection for growth with either methane- or propane-oxidation. The overall goal of the present study was to extend the range of microbial environments for which the biomarker techniques have been validated to include surface and subsurface soils in which methane- and propane-oxidizing bacteria are enriched.

3. MATERIALS AND METHODS

3.1. Materials

Solvents were highest quality distilled in glass and were of residue analysis grade or better (J.T. Baker Chemical Co., Phillipsburg, NJ). Standards and derivatizing agents were purchased from Supelco, Inc. (Bellefonte, PA), Applied Science (State College, PA), Aldrich, Inc. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), and Pierce Chemical Co. (Rockford, IL).

3.2. Soil and microcosm samples

Soil was recovered from a shallow semi-confined alluvial aquifer at the Stanford University field demonstration site at Moffett Naval Air Station, Mountain View, CA, [8]. The aquifer zone consists of a layer of poorly sorted silty sand and gravel approximately 1.18 to 1.8 m thick bounded above and below by clay. A series of injection and extraction wells were drilled and the sediments recovered for microcosm studies. This core material was transferred to the R.S. Kerr Laboratory, Ada, OK, where it was packed into glass columns (3.7 cm diameter × 22 cm long), perfused with Moffett Field site water, sparged with oxygen, amended with an aqueous solution of both trichloroethylene (TCE) and 1,1,1,-thrichloroethane, and an aqueous solution saturated with either methane or propane. A treatment cycle consisted of perfusion of two pore volumes (150 ml) of solution and incubation for 10 days followed by flushing with two pore volumes. After eleven cycles

the sediment was lyophilized and sent to Knoxville for analysis. Control samples of untreated Moffett Field subsurface aquifer material similar to that used in the microcosm experiments were also obtained.

Experimental verification of biodegradability of TCE with concomitant use of hydrocarbons was performed as described [8]. Controls for microbial activity were poisoned with 0.1% sodium azide in the perfusate.

In other experiments a prototype soil reactor was constructed at Racine, WI, using Rollin muck soil (6.5% organic matter, 45% clay with a pH of 7.4) through which a mixture of air with 0.2% of hydrocarbons with 27 mol% propane, 29 mol% iso-butane, and 44 mol% *n*-butane flowed at 3 cm^3 per cm^2 surface area per min [9]. In the soil reactor, the soil was 90 cm thick and covered an area of 190 m^3. The pressure drop across the 90 cm thick bed was 85 cm water and the residence time of the perfused gases in the soil was approximately 15 min. Samples were recovered at levels of 15, 59, and 88 cm below the surface, packed in ice, and sent to Knoxville for analysis. The soil reactor has been exposed to the hydrocarbons for three years. Control samples were recovered from Rollin muck soil not exposed to the hydrocarbons.

The removal of TCE in acclimated Rollin muck soil was examined by adding 46 g of muck soil at 30% moisture content to replicate 160 ml serum bottles with Teflon-faced silicone septa and aluminum crimp caps. The air-filled space in the bottle was 60-times greater than the air-filled pores of the soil in the prototype bioreactor. This dilution slowed the rate of TCE biodegradation sufficiently to provide accurate estimations. Each measurement represented a microcosm sampled for alkanes in the head space, adding water to form a slurry with the soil and then collecting the TCE for gas chromatographic analysis by the purge and trap technique [9].

3.3. Organisms and cultural conditions

Propane and methane-oxidizing organisms were isolated from the Rollin muck soil by enrichments in 150 ml serum bottles with 2 g soil plus 50 ml of mineral salts medium with a headspace containing roughly 50% methane or propane and air. The bottles were shaken at 100 r.p.m. After about a month at room temperature 0.1 ml was plated on mineral salts medium on Nobel agar (Difco Inc., Detroit, MI). Isolates were restreaked for purity and single colony isolates grown for analysis of the PLFA. The mineral salts medium contained in g/l: 2.0 $NaNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g KCL, 0.015 g $CaCl_2$ vitamins and 2 ml of trace minerals. The trace mineral solution contained in g/l: 1.5 g nitrilotriacetic acid, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4\ H_2O$, 0.17 g $CoCl_2 \cdot 2H_2O$, 0.1 g $CaCl \cdot 2H_2O$, 0.1 g $ZnCl_2$, 0.02 g $CuCl_2 \cdot 2H_2O$, 0.01 g H_3BO_3, 0.01 g Na Molybdate, 1.0 g NaCl, 0.017 g Na_2SeO_3, 0.026 g $NiSO_4 \cdot 6H_2O$. The vitamin solution in g/l contains 0.002 g biotin, 0.002 g folic acid, 0.01 g pyridoxine · HCl, 0.005 g thiamine · HCl, 0.005 g riboflavin, 0.005 g pantothenic acid, 0.0001 g cyanocobalamin, 0.005 g *p*-aminobenzoic acid, and 0.005 g lipoic acid.

Organisms were harvested by centrifugation at $10000 \times g$ for 15 min at 4°C, washed twice in phosphate buffer or growth medium minus energy source, and lyophilized.

3.4. Extraction

The analytical sequence utilized for PLFA analysis involves extraction, fractionation on silicic acid, derivatization, and analysis by capillary gas chromatography (GC) with mass spectral structural confirmation. The modified one-phase Bligh and Dyer extraction was utilized for all samples [10]. Duplicate samples were extracted separately and all data is expressed as the mean of two determinations unless otherwise stated. After the overnight separation of the lipid and aqueous phases in the second stage of the extraction, the organic fraction was filtered through fluted Whatman 2V filters pre-extracted with chloroform and reduced in volume using a rotating evaporator. The samples were transferred to teflon lined screw capped test tubes and dried under a stream of nitrogen at room temperature.

3.5. Polar lipid isolation

Silicic acid columns were prepared using 1 g Unisil (100–200 mesh, Clarkson Chemical Co., Inc, Williamsport, PA) activated at 100°C for 60

min and pre-extracted with chloroform. Columns were prepared with the approximate ratios of 50 : 1 stationary phase to lipid (dry weight) and 1.7 : 1 stationary phase column bed height : cross sectional area. Total lipid was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 10 ml of chloroform, acetone, and methanol eluted the neutral, glyco- and polar lipids. The polar lipid fraction was dried under a stream of nitrogen.

3.6. Mild alkaline methanolysis

The mild alkaline methanolysis procedure [10] was utilized to prepare methyl esters of the ester-linked fatty acids of the polar lipids.

3.7. Determination of mono-unsaturated PLFA double bond position and configuration

The dimethyl disulfide (DMDS) adducts of mono-unsaturated PLFA were formed to determine double bond position and geometry using methods previously described [11–13]. Samples were analyzed by capillary GC and GC/MS as described below. These derivatives increase the resolution between *cis* and *trans* geometrical isomers in capillary GC.

3.8. Gas chromatography (GC)

Dry PLFA methyl esters were dissolved in isooctane containing the internal standard of methyl nonadecanoate. Samples of 1.0 μl were injected onto a 50 m nonpolar, cross-linked methyl silicone fused silica capillary column (0.2 mm i.d., Hewlett Packard) in a Shimadzu GC-9A GC. A 30 s splitless injection with the injection temperature at 270 °C was used. Hydrogen at a linear velocity of 35 cm/s was the carrier gas with a temperature program starting with an initial temperature of 80 °C. After a one minute delay at 80 °C, the temperature was programmed at a 10 °C/min rise to 150 °C, followed by a 3 °C/min rise to 240 °C, followed by a 5 °/min rise to 280 °C with a terminal isothermal period of 5 min at 280 °C. Detection was by hydrogen flame (F.I.D.) using a 30 ml/min nitrogen make up gas at a temperature of 270 °C. An equal detector response was assumed for all components. Peak areas were quantified with a programmable laboratory data system (Nelson Analytical 3000 Series Chromatography Data System (Revision 3.6)).

3.9. Gas chromatography / mass spectrometry (GC / MS)

Tentative component identification prior to GC/MS analysis was based on comparison of the retention time data or co-elution with authentic standards. GC/MS analysis was performed on a Hewlett Packard 5996A GC/MS fitted with a direct capillary inlet utilizing the same chromatographic system except for use of a helium carrier gas and the temperature program which was begun at 100 °C and increased to 280 °C at 3 °C/min for a total analysis time of 60 min. The electron multiplier voltage was between 1800 and 2000 V, the transfer line maintained at 300 °C, the source 280 °C and analyzer 250 °C, and the GC/MS was autotuned with DFTPP (decafluorotriphenylphosphine) at m/z 502 with an ionization energy of 70 eV. The data was acquired and manipulated using the Hewlett Packard RTE 6/VM data system. Other conditions were as described previously [14].

3.10 Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms: number of double bonds with the position of the double bond nearest to the aliphatic (w) end of the molecule indicated. This is followed by the suffix c for *cis* and t for *trans* configuration of monoenoic fatty acids. The prefixes i, a, or br indicate iso, anteiso, or branched (position undetermined). Mid-chain branching is indicated by the number of carbon atoms from the carboxyl end of the molecule and Me for the methyl group (10Me16 : 0 is a 17-carbon PLFA). Cyclopropane rings are indicated with the prefix cy with the ring position indicated from the Aliphatic end of the molecule.

4. RESULTS

4.1. Effect of amending subsurface materials with propane and a mixture of methane and propane

The subsurface sediments from the aquifer at the Moffett Field demonstration site showed a

PLFA profile typical of subsurface sediments with high levels of saturated, branched, cyclopropane, and monoenoic PLFA (Table 1, first column). Exposing the sediments to propane and air and then sealing the system for 10 days through a series of 13 cycles depressed the total biomass by a factor of 10 and profoundly shifted the proportions of PLFA (Table 1, second column). There was a 7-fold increase in the proportion of 18 : 2w6, a 4-fold increase in 18 : 1w9c, but little change in the proportions of 16 : 0. There was a 7-fold drop in the proportions of 10Mel6 : 0. The most striking change was the appearance of an unusual PLFA, 16 : 1w6c, which became the most prominent component of the polar lipids. Two other unusual PLFA also increased in their proportions, 10Me18 : 0, and 16 : 1w5c. Exposing the sediments to methane for 12 cycles but with an inadvertent exposure to propane for one cycle showed a higher total biomass (roughly half the starting material) and decreases in 16 : 1w6c and 10Me18 : 0 over amounts found in the propane exposed material. Methane exposure also resulted in the appearance of the PLFA, 18 : 1w8c which has been shown to be characteristic of authentic type II methane-oxidizing bacteria [14]. The data given in Table 1, the second and third columns, show no standard deviations as the total combined sample was utilized to provide enough material for mass spectral confirmation of the structure. In separate experiments the standard deviation ranged between 10 and 50% of the mean ($n = 3$) in these samples. In these experiments the oxygen utilization in the microcosms acclimated to propane removed 54–64% of the theoretical oxygen demand of the propane removed from the perfusion water [8]. The oxygen depletion in the columns acclimated to methane was 91–107% of the theoretical oxygen demand [8].

Table 1

Effect of propane and methane + a single exposure to propane amendment on phospholipid ester-linked fatty acid (PLFA) profile of the microbiota in sub-surface materials from the Moffett Field, CA field demonstration site

PLFA	Mol %			
	A		B	C
	Control [b]	(S.D.)	Propane [c]	Propane and Methane [d]
14:0 [a]	hd [g]	–	nd	0.8
i15:0	1.6	0.0	nd	3.0
a15:0	1.6	0.07	nd	2.4
15:0	0.8	0.14	1.5	0.8
i16:0	4.3	0.14	nd	2.0
16:1w7c	3.5	–	nd	1.5
16:1w6c	nd	–	21.9	17.7
16:1w5c	1.7	0.21	3.2	2.3
16:1w5t	nd	–	nd	6.4
16:0	15.2	0.21	16.6	15.9
i17:1w7c	1.9	0.07	2.2	2.1
10Me16:0 [a]	13.3	0.0	1.2	7.0
i17:0	4.4	0.21	nd	1.7
a17:0/17:1	2.9	0.07	4.4	1.9
cy17:(7,8)	2.0	0.42	nd	0.5
17:0	1.0	0.0	3.2	0.9
18:2w6 [a]	0.9	0.14	7.2	1.1
18:1w9c	4.5	0.42	18.4	3.3
18:1w8c	nd	–	nd	11.7
18:1w7c	6.3	0.84	nd	0.2
18:1w5c [a]	1.0	0.14	nd	0.3
18:0	4.9	0.21	5.3	2.2
10Me18:0	2.7	0.07	8.6	1.7
19:1 [a]	nd	–	1.7	0.7
cy19:0	5.6	0.07	3.3	9.6
i20:0 [a]	nd	–	nd	0.2
20:1 [a]	nd	–	nd	0.2
20:1 [a]	nd	–	nd	0.1
20:0 [a]	1.7	1.1	1.2	1.9
22:0 [a]	1.5	0.71	nd	0.0
pmol/g dwt [e]	556 [f]	67.9	57	253

[a] Identification based on retention time on capillary gas chromatography only.
[b] Subsurface materials from Moffett Field demonstration site. Data given as a mean (S.D.) $n = 3$.
[c] Material from Column A exposed to propane.
[d] Material from Column A exposed to methane + propane.
[e] Total PLFA pmol/g dry wt.
[f] Additional PLFA (not shown) accounts for missing mol %.
[g] nd indicates not detected.

4.2. *Effect of exposing Rollin muck soil to a mixture of hydrocarbons*

Table 2 gives the mean and standard deviation of the proportions of PLFA found in the control and the Rollin muck soil exposed to a mixture of propane, *iso*-butane, and butane over a period of 2 years. The gassed soils show a consistently higher biomass based on the total PLFA/g dry wt and a higher proportion of the endogenous storage lipid PHA relative to the PLFA recovered from the soil. Highest levels were found at depths of 14.7 and 59

Table 2

Proportions of phospholipid ester-linked acids (PLFA), total biomass, and poly beta-hydroxyalkanote (PHA) from the microbiota induced by exposure to short chain hydrocarbons in a soil bed reactor; air was injected at a depth of 90 cm

PLFA	Mol % [a]					
	Gassed			Not Gassed		
	88 cm	59 cm	14 cm	88 cm	59 cm	14 cm
i15:1	0.3 (0.1)	0.7 (0.0)	0.6 (0.0)	0.8 (0.0)	0.7 (0.2)	0.8 (0.2)
i15:0	3.6 (1.0)	3.4 (0.0)	4.4 (0.0)	6.6 (0.0)	6.3 (0.9)	6.0 (0.4)
a15:0	2.9 (0.7)	2.8 (0.1)	3.2 (0.1)	5.2 (0.3)	5.0 (0.8)	6.8 (0.4)
15:1w6	0.2 (0.1)	0.3 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.5 (0.0)
Br16:0	0.3 (0.0)	0.2 (0.0)	0.4 (0.0)	0.7 (0.1)	0.6 (0.0)	0.8 (0.1)
il6:1w6	0.5 (0.1)	0.6 (0.0)	0.7 (0.0)	0.6 (0.0)	0.8 (0.0)	0.7 (0.0)
10Me15:0	0.3 (0.0)	0.2 (0.0)	0.3 (0.0)	0.3 (0.0)	0.5 (0.0)	0.3 (0.1)
il6:0	2.4 (0.0)	2.1 (0.0)	2.1 (0.0)	2.4 (0.0)	2.6 (0.3)	2.2 (0.0)
16:lwll,w9	2.0 (0.2)	2.5 (0.0)	2.1 (0.1)	1.7 (0.0)	1.9 (0.1)	1.6 (0.2)
16:1w7c	7.1 (0.1)	6.8 (0.0)	9.3 (0.4)	8.1 (0.1)	6.6 (0.1)	7.3 (0.2)
16:1w6c	2.9 (0.1)	4.8 (0.4)	1.6 (0.1)	0.4 (0.0)	0.4 (0.0)	0.3 (0.1)
16:1w5c	6.3 (0.6)	14.3 (0.0)	4.3 (0.2)	4.5 (0.2)	4.2 (0.2)	4.4 (0.05)
16:0	13.1 (1.1)	11.5 (1.0)	10.1 (0.3)	10.5 (0.1)	10.1 (0.8)	8.7 (0.02)
Br17:0	0.9 (0.1)	0.5 (0.1)	1.0 (0.1)	1.1 (0.1)	1.6 (0.4)	1.2 (0.02)
i17:1w7	2.6 (0.2)	1.8 (0.1)	3.3 (0.1)	4.3 (0.1)	4.4 (0.0)	5.6 (0.02)
10me16:0	5.4 (0.1)	3.3 (0.2)	6.5 (0.3)	10.9 (0.2)	13.3 (1.4)	9.6 (0.04)
i17:0	1.3 (0.1)	0.9 (0.0)	1.3 (0.0)	1.7 (0.0)	1.7 (0.1)	1.8 (0.1)
a17:0/17:1w8	2.1 (0.1)	1.7 (0.0)	2.1 (0.0)	2.5 (0.0)	2.5 (0.1)	2.3 (0.0)
17:1w6(cyl7:0w7,8)	3.3 (0.1)	2.8 (0.1)	4.9 (0.3)	4.4 (0.1)	4.3 (0.5)	4.0 (0.1)
cyl7:0	1.0 (0.1)	2.5 (0.0)	0.4 (0.0)	0.4 (0.1)	0.5 (0.1)	0.4 (0.0)
17:0	0.8 (0.1)	0.8 (0.0)	0.6 (0.0)	0.6 (0.0)	0.5 (0.0)	0.5 (0.0)
5me17:0	0.9 (0.1)	0.5 (0.0)	1.3 (0.0)	1.7 (0.1)	1.8 (0.2)	2.5 (0.3)
10me17:0	0.8 (0.1)	0.5 (0.0)	0.9 (0.1)	0.8 (0.0)	0.8 (0.0)	1.2 (0.1)
Br18:1	0.4 (0.0)	0.7 (0.1)	0.8 (0.2)	0.3 (0.1)	0.3 (0.0)	0.3 (0.0)
18:1w7c/18:1w8c	10.0 (0.4)	7.9 (0.3)	10.5 (0.1)	6.1 (0.1)	5.5 (0.7)	7.4 (0.3)
18:0	2.0 (0.4)	1.2 (0.0)	1.8 (0.0)	2.2 (0.1)	2.3 (0.1)	1.9 (0.1)
11me18:1w6	1.0 (0.2)	0.6 (0.0)	0.9 (0.0)	0.4 (0.0)	0.4 (0.1)	1.6 (0.0)
10me18:0	5.2 (1.0)	5.8 (0.4)	2.6 (0.3)	1.6 (0.1)	1.5 (0.1)	1.6 (0.1)
20:4w6	0.6 (0.1)	0.5 (0.0)	0.1 (0.0)	0.2 (0.1)	0.2 (0.1)	0.4 (0.0)
20:1w9	0.6 (0.0)	0.4 (0.0)	0.8 (0.0)	0.6 (0.0)	0.8 (0.1)	0.6 (0.1)
20:0	0.5 (0.2)	0.3 (0.0)	0.5 (0.0)	0.8 (0.1)	0.8 (0.1)	0.7 (0.2)
21:0	0.6 (0.2)	0.3 (0.0)	0.5 (0.0)	1.0 (0.0)	0.7 (0.3)	0.9 (0.1)
PLFA [b]	42.4 (3.4)	78.6 (4.6)	48.9 (10.8)	31.2 (4.9)	28.7 (4.4)	23.7 (2.0)
PHA [c]	85 (120)	705 (742)	55 (7)	10 (14)	10 (14)	20 (28)

[a] Data are given as mean mol % (S.D.), $n = 5$, with PLFA indicated as defined in the text. Only those PLFA showing significant difference ($P > 0.05$) by ANOVA are given in the table.

[b] Total nmoles PLFA/g dry wt.

[c] Total nmoles PHA/g dry wt.

cm below the surface. Maximum rates of destruction of the alkane occurred at the 52 cm depth which was just above the water level. The lowest increase in biomass occurred at the 88 cm layer which was very close to the level at which the gases and air were injected into the soil bioreactor. Only the PLFA with a statistical difference by ANOVA $P > 0.05$ are listed in the table. Subjecting the data of Table 2 to be a Tukey's significance difference test shows significant increases in the proportions of 16:1w6c as consistently higher in all the soils exposed to propane, *iso*-butane, and butane (Table 3). The combined 18:1w7c + 18:1w8c and 10Me18:0 PLFA showed signifi-

Table 3

Tukey's Significant Difference Maps generated from Tukey's HSD test (SPSS), with the within-experiment, family-wise error rate set at alpha = 0.05 for logarithmically-transformed data from Table 2. Treatment means for each PLFA increase left to right, and those means connected by a common line segment are not significantly different for this test. Abbreviations as in Table 1.

i15:1w10	1A [a]	3A	2B	2A	1B	3B
i15:0	2A	1A	3A	3B	2B	1B
a15:0	2A	1A	3A	3B	2B	1B
15:1w6	3B	2B	3A	1A	1B	2A
br16:0	2A	1A	3A	2B	1B	3B
i16:1w6	1A	1B	2A	3B	3A	2B
10Me15:0	2A	1B	1A	3B	3A	2B
i16:0	3A	2A	3B	1B	1A	2B
16:1w11c/16:1w9c	3B	1B	2B	1A	3A	2A
16:1w7c	2B	2A	1A	3B	1B	3A
16:1w6c	3B	2B	1B	3A	1A	2A
16:1w5c	2B	3A	3B	1B	1A	2A
16:0	3B	3A	2B	1B	2A	1A
br17:0	2A	1A	3A	1B	3B	2B
i17:1w7c	2A	1A	3A	1B	2B	3B
10Me16:0	2A	1A	3A	3B	1B	2B
i17:0	2A	1A	3A	2B	1B	3B
a17:0/17:1w8c	2A	1A	3A	3B	1B	2B
17:1w6/cy17:0(7,8)	2A	1A	3B	2B	1B	3A
cy17:0(5,6)	3A	1B	3B	2B	1A	2A
17:0	2B	3B	3A	1B	2A	1A
10Me17:0	2A	1A	2B	1B	3A	3B
br18:1	2B	3B	1B	1A	2A	3A
18:2w6	1A	2A	2B	1B	3B	3A
18:1w7c+18:1w8c	2B	1B	3B	2A	1A	3A
18:0	2A	3A	3B	1A	1B	2B
11Me18:1w6	2B	1B	2A	3B	3A	1A
10Me18:0	2B	1B	3B	3A	1A	2A
20:4w6	2B	1B	3B	2A	1A	3A
20:1w9	2A	1A	3B	1B	2B	3A
20:0	2A	3A	1A	3B	1B	2B
21:0	2A	3A	1A	2B	3B	1B

[a] 1A, 2A, 3A indicate samples exposed to propane, butane and isobutane recovered from 88 cm, 59 cm, and 14.7 cm below the surface of the soil bed bioreactor. 1B, 2B and 3B are from the same depths in a soil sample not exposed to the hydrocarbons.

cantly higher proportions in two of the gassed soils (Table 3).

Microcosms formed of Rollin muck soils exposed to butane showed concomitant degradation of TCE and butane that was not detected in the control (Figure 1). The control had no oxygen in the headspace.

4.3. PLFA patterns of monocultures isolated with propane or methane on mineral-salts agar

Table 4 shows the PLFA pattern of 4 monocultures grown from single colony isolates grown with propane and 2 grown with methane recovered from the Rollin muck soil. The propane isolates formed colored colonies with the microscopic appearance of Actinomycetes. These monocultures all showed high proportions of the unusual PLFA (16:1w6c) seen in both soils exposed to propane. These bacteria also showed high proportions of tuberculostearic acid, 10Me18:0 a characteristic PLFA of many Actinomycetes [15]. The cyclopropane PLFA cy17:0 or cy19:0 were not detected in organisms grown with propane. The isolates also showed high proportions of

17 : 1w8c which was not detected as a significant PLFA in the subsurface aquifer material or muck soil exposed to propane.

When methane was utilized as carbon source in mineral salts agar, pink colonies typical of type II methane oxidizers were isolated. These monocultures displayed high proportions of the unusual PLFA, 18 : 1w8c which to date has only been found in type II methane-oxidizing bacteria [14–16] and that has been found consistently when soils are exposed to air and methane [17]. The PLFA representative of propane-utilizing isolates were not detected in these methane-utilizing organisms (Table 4).

4.4. Mass spectral identification of characteristic PLFA of methane and propane utilizing bacteria

The characteristic ion fragments of the adducts formed by reaction of DMDS with the 'signature' PLFA found in the propane- and methane-utilizing bacteria stimulated in subsurface materials and muck are given in Table 5. Iso or mid-chain

Table 4

Phospholipid ester-linked fatty acid patterns of monocultures grown with propane (Columns A–D) or methane (Columns E, F) isolated from Rollin Muck soil

PLFA	Mol %					
	Propane-utilizing				Methane-utilizing	
	A	B	C	D	E	F
14 : 0	0.8	0.4	0.7	0.6	nd	nd
15 : 1	0.2	0.2	0.2	0.2	nd	nd
15 : 1w5c	0.1	0.1	nd	Trace	nd	nd
15 : 1	0.7	0.9	nd	0.7	nd	nd
15 : 0	5.8	4.5	15.7	5.3	nd	nd
16 : 1w9c	0.5	0.3	nd	0.3	nd	nd
16 : 1w7c	0.7	17.4	0.7	0.7	0.6	2.9
16 : 1w6c	8.1	7.4	9.4	7.9	nd	nd
16 : 1w5c [a]	nd	Trace	0.2	nd	nd	nd
16 : 0	23.6	20.7	11.4	25.4	0.2	0.2
i17 : 1 [a]	nd	nd	nd	nd	nd	Trace
10Me16 : 0 [a]	Trace	Trace	1	Trace	nd	nd
17 : 1w8	13.2	9.5	13.6	11.2	0.2	0.2
17 : 1w7	2.9	2	4.7	2.5	0.1	0.1
i17 : 0	nd	nd	nd	nd	0.1	0.1
17 : 1w6 [a]	nd	0.1	0.1	0.1	nd	nd
cy17 : 0	Trace	1	0.6	0.1	nd	nd
17 : 0	4.6	3.9	6.2	4.8	0.1	Trace
i18 : 0	3.5	1.7	24.9	3.1	nd	nd
18 : 1w9c	21.9	13.3	2.4	20.2	nd	2.8
18 : 1w8c	nd	Trace	0.4	nd	22.9	18.9
18 : 1w7c	0.1	9.9	0.1	1.7	75.4	74.6
18 : 1w5c [a]	nd	Trace	nd	nd	0.1	0.1
18 : 0	1.1	1.5	Trace	1.3	0.1	Trace
br19 : 1	nd	nd	nd	0.9	nd	nd
10Me18 : 0	10.5	4.4	6.8	11.5	nd	nd
19 : 1	1.3	0.6	0.9	1	nd	nd
cy19 : 0	nd	nd	nd	0.1	nd	nd
Total PLFA	78.9	98.6	35.8	72.7	179.8	208.0

[a] Identification based on GC retention time only. nmol/g dry wt.

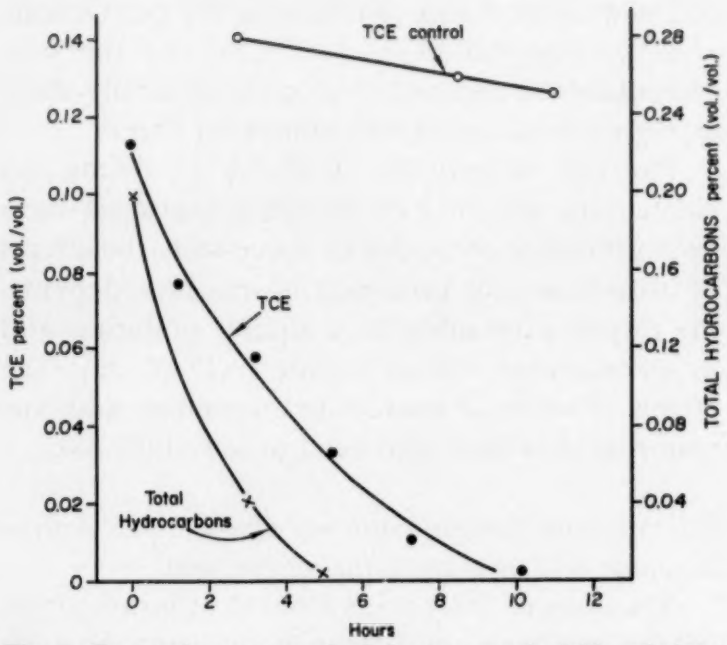

Fig. 1. Concomitant removal of the vapors of trichlorethylene and a mixture of propane, *n*-butane and isobutane in serum bottle microcosms constructed with Rollin muck soil. Conditions of the experiment are as described [9].

Table 5

Structural characterization of 'signature' PLFA from methane-(M) and propane-(P) utilizing bacteria by electron impact mass spectrometry

Mono-unsaturated fatty acids [a]	M+	w-fragment [b]	Δ-fragment [c]	Type
15:1w5	348(26.7) [d]	117(88.9)	231(100)	P
16:1w9	362(28.8)	173(74.2)	189(46.7)	P
16:1w6	362(22.9)	131(89.2)	231(100)	P
17:1w8	376(20.6)	159(82.8)	217(100)	P
17:1w7	376(43.9)	145(64.5)	231(99.9)	P
18:1w8	390(16.4)	159(78.0)	231(100)	M
18:1w7	390(11.0)	145(60.2)	245(83.0)	M

Other fatty acids [e]	M+	diagnostic ions	Type
i18:0	298 (8.5)	225(22.9), 43(60.7)	P
10Me18:0	312(12.3)	171 (7.8), 199(15.3)	P

[a] Identified as dimethyl disulfide adduct.
[b] w-fragment indicates fragment including aliphatic end of molecule.
[c] Δ-fragment indicates fragment including carboxylic end of molecule.
[d] Fragment masses given as Mass (data given as M/Z abundance).
[e] Identified as fatty acid methyl esters.

branching is defined by the fragmentation patterns given in the lower section of Table 5.

5. DISCUSSION

5.1. Usefulness of Biomarkers to define shifts in microbial community structure

Assessment of the microbial community found in soils and sediments presents complex assay problems. Determination of biomass by classical viable counts methods require that microbes be quantitatively recovered from the substratum and that the growth medium be universal. Direct microscopic estimation of biomass are difficult, particularly in the surface soils where there is great diversity in the microbial morphology and assumptions of biovolumes can be complex. Use of biomarker components to estimate biomass requires only that the component be universally distributed, be reproducibly related to the cell biomass, and be quantitatively recoverable. The biomarker components universally distributed in microbes such as intercellular ATP, cell wall muramic acid and polarlipid phosphate and ester-linked fatty acids have been shown to give identical biomass and numerical estimations in uncontaminated subsurface soils as the direct microscopic counts [3]. In the soil materials analyzed in this study, the microbiota consisted of uniformly sized small coccibacillary bacteria and the biovolume was readily estimated.

If specific biomarker component patterns can be related to specific subsets of the microbial community, then the patterns can be used to define the microbial community structure. PLFA are presently the most sensitive and useful chemical measures of community structure [17]. The validation of the use of biomarkers as 'signatures' for particular groups of organisms has been based on the induction of shifts in microbial community structure by altering the microenvironment with resulting changes that can often be predicted (such as the formation of fungus 'heaven' and 'hell' on biofilms suspended in a subtropical estuary by manipulation of nutrients and inhibitors) [18]. A second validation rests on the alteration of community structure by predator grazing with known

specificity (such as selective removal of non-photosynthetic microeukaryotes from sandy sediments by sand dollars) [19]. Possibly the best validation is the detection of biomarkers for specific microbes in the environment that can be found in specific isolates from that environment. The present study extends this validation to the alkane-oxidizing bacteria in surface soils and aquifer materials.

In these studies the position and confirmation of unsaturation of the monoenoic PLFA have been important. Gillan and Hogg [20] used the position of unsaturation in monounsaturated FAME to classify sedimentary microorganisms into 'chemotypes'. Nichols et al. [14,21] found unusual patterns of unsaturation in the monoenoic PLFA of the methane-oxidizing bacteria and the pathogen *Francisella tularensis.* With both the acid-forming *Thiobacilli* [22], and the sulfate-reducing bacteria [23–26], the monoenic PLFA analysis was very important. These 'signature' patterns were sufficiently unique for detection of these organisms in environmental samples [27,28]. Guckert et al. [5] used changes in the proportions of monoenoic PLFA with defined positions of unsaturation to indicate the shifts in the benthic marine microbiota from aerobic to anaerobic growth.

The PLFA analysis not only provides insight into the microbial community structure but also the nutritional status. Shifts in PLFA pattern occur in some bacteria with culture conditions. Cyclopropane rings are formed from monoenoic esters of specific phospholipids in the membranes of bacteria [29]. Their formation with the concomitant decrease in monoenoic PLFA occurs in monocultures that undergo metabolic stress such as stationary phase growth [30]. The same phenomenon has been observed for the benthic marine microbiota [31]. Guckert et al. [32] showed that accumulation of *trans* monoenoic PLFA correlated with the starvation response in *Vibrio cholerae.* Similar accumulations of *trans* monoenoic PLFA occurred in subsurface bacteria grown under conditions of starvation (Pfiffner, unpublished observations). Shifts in PLFA in some bacteria also have been detected when the major carbon source is changed. Carbon sources with odd numbered chains can increase the proportions of odd numbered PLFA [25,26,33] and the soils and organisms exposed to propane generally show higher proportions of odd numbered PLFA.

The use of patterns of PLFA to define the community structure of microbial consortia have been utilized to show detrial succession, the effects of disturbance, or predation in marine sediments, the response to subsurface aquifer pollution and in environment effects testing [2,17,34–36]. The effects of shifts in microbiota biofouling and corrosion studies have also been reviewed [37,38].

5.2. Structural identification of unusual PLFA from propane- and methane-utilizing bacteria

The unusual fatty acids from the phospholipids of the methane- and propane-utilizing bacteria were tentatively identified from their retention times when compared to authentic standards. To define the structure of the PLFA, derivatives were made and the structures confirmed based on interpretation of their fragmentation patterns obtained with electron ionization MS (Table 5).

The position of unsaturation in monoenoic PLFA was the key to defining the 'signature' PLFA biomarkers for the methane- and propane-utilizing bacteria that were induced by exposure of soils to the hydrocarbons. Unsaturations at the w6 position in the 16 : 1w6c found in high proportions in the propane-utilizing bacteria and at the w8 position in the 18 : 1w8c of the methane-oxidizing bacteria are sufficiently unusual to be diagnostic in environments as complex as surface soils and aquifer materials. High proportions of 16 : 1w6c in the PLFA are not unique to propane-oxidizing bacteria. This PLFA is also found in type I methane-oxidizing bacteria [14,16,39]. However, the high proportion of 10Me18 : 0 PLFA (characteristic of many Actinomycetes) [15] and 17 : 1 isomer present in the propane-oxidizing bacteria found in this environment are not found in the type I methane-oxidizing bacteria. Based on the differences in PLFA profiles like those observed in this study, it should be possible to use these methods to monitor shifts in microbial biomass and community structure in aquifers where indigenous bacteria are stimulated to transform pollutant compounds.

DISCLAIMER

Although the research in this article has been funded wholly or in part by the United States Environmental Protection Agency, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

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Competition for nitrate between denitrifying *Pseudomonas stutzeri* and nitrate ammonifying enterobacteria

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1. SUMMARY

Competition for nitrate between nitrate ammonifying enterobacteria and a denitrifying pseudomonad was studied in electron acceptor-limited chemostats. In pure cultures, using different carbon and energy sources, the C/N-ratio needed for denitrification is far lower than that required for nitrate ammonification. In mixed cultures of *Citrobacter freundii* and *Pseudomonas stutzeri*, competing for nitrate with L-lactate as electron donor, the nitrate ammonifying organism dominated at dilution rates of $D \leq 0.14\ h^{-1}$. Competition for both nitrate and L-lactate at a dilution rate of $D = 0.05\ h^{-1}$ always resulted in the coexistence of both species. Using glucose as additional carbon source, the final ratio of nitrate ammonifying and denitrifying organism depended on the C/N-ratio as well as on the dilution rate. The results of the study are discussed with respect to field data.

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2. INTRODUCTION

In anaerobic ecosystems, nitrate can be used by a wide variety of facultative and strictly anaerobic bacteria as a terminal respiratory electron acceptor. Depending on the end-products, two different pathways of dissimilatory nitrate reduction are distinguished: (i) denitrification, carried out by oxidative bacteria such as *Pseudonomas* sp., leads predominantly to gaseous products as nitrous oxide and molecular nitrogen [1]; (ii) alternatively, fermentative bacteria such as *Klebsiella, Vibrio* and *Aeromonas*, but also clostridia and *Desulfovibrio* sp. reduce nitrate to nitrite and subsequently to ammonia [2–6]. This process is called 'dissimilative nitrate reduction' or 'nitrate ammonification'. Field studies have shown that in soils [7], estuarine and marine sediments [8,9] and lake sediments [10,11] 20–70% of N-15 labelled nitrate is reduced to ammonia as a consequence of nitrate ammonification. King and Nedwell [12] recently demonstrated with sediment slurries prepared from estuarine samples that at low nitrate concentrations reduction to ammonia was of the same order of magnitude as denitrification, whereas at high nitrate concentrations nearly all the nitrate was reduced to gaseous products. Previously, the same authors suggested that in sedi-

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ments, exposed for long periods to different nitrate concentrations, the composition of the bacterial-nitrate reducing community and therefore also the balance of the end products of nitrate dissimilation was dependent on nitrate load [13].

Competition between denitrification and dissimilatory nitrate reduction to ammonia has been analysed theoretically by Tiedje et al. [14] using Michaelis-Menten models. The evaluation was limited to non-growth conditions and a few kinetic parameters. Here we report on our chemostat studies of nitrate reduction by two *Klebsiella* sp. and *Citrobacter freundii* and of the competition of the latter with *Pseudomonas stutzeri* for nitrate.

3. MATERIALS AND METHODS

3.1. Source of organisms

P. stutzeri DSM 50238, *C. freundii* DSM 30039 and *K. terrigena* DSM 2687 were obtained from the Deutsche Sammlung für Mikroorganismen, Braunschweig, F.R.G. *Klebsiella* sp. strain K312 was obtained from Dr. G.M. Dunn, Fermentech. Ltd., Edinburgh, U.K.

3.2. Media and cultivation

The bacteria were maintained on agar slants with the medium described by King et al. [15]. For chemostat experiments, a simple mineral salts medium [16] was used, with the concentration of all nutrients lowered by 50%; NH_4Cl was omitted. Glucose, L-lactate and KNO_3 were added as indicated in the experiments described.

Continuous cultures (700 ml) were run in a 1-liter laboratory fermentor at 30 °C. The pH was maintained at 7.4 by automatic addition of sterile 4 M NaOH. Anaerobiosis was ensured by flushing the medium reservoir and chemostat vessel with oxygen-free nitrogen gas at a rate of 800 ml/h. All tubing was of oxygen-impermeable rubber.

Competition experiments were started with pure cultures pregrown in nitrate limited chemostats (5 mM KNO_3) at a dilution rate of $D = 0.1\ h^{-1}$ using the same carbon and energy source. Competition was followed by differentiating and counting colonies on the appropriate agar media. 100 µl portions of the diluted samples were spread over agar plates containing defined medium [16] supplemented with 0.5% (w/v) starch and 1.2% (w/v) purified agar (Merck), or nutrient rich King B medium [15]. The agar plates were incubated aerobically at 30 °C. *Citrobacter* colonies appeared after 1 and 5 days on complex and defined media, respectively. *Pseudomonas* colonies could be counted after 2 days on both media. When growing on starch agar, the pseudomonad was identified by a zone of hydrolysis around the colonies. For each determination at least 500 colonies were counted.

3.3. Enzymes assays

Nitrate reductase activity ($Na_2S_2O_4$-benzyl-viologen-linked) was determined in soluble and particulate fractions of cell extracts as described in [17]. Nitrite reductase was only detected and assayed in soluble fractions [18].

3.4. Chemical analyses

Nitrite was assayed colorimetrically [4], ammonia by enzymatic techniques [4]. Glucose and nitrate were determined enzymatically using test-kits of Boehringer, Mannheim, F.R.G. Ethanol, acetate and lactate were analysed with a Packard 430 gas chromatograph equipped with a flame ionisation detector according to [19,20]. Protein in cell-free extracts was assayed according to the method of Bradford [21]. Bacterial biomass was determined by dry-weight measurements.

3.5. Other methods

Maximal specific growth rates were determined by calculating the difference between dilution rates and the recorded washout rate according to [22]. The substrate saturation constants were calculated from the μ_{max} values and the concentrations of the growth-limiting substrate at various dilution rates using the Monod equitation as described in [23].

Nitrate and nitrite were determined in samples of culture fluid, having been passed through a membrane filter (0.20 µm pore size) directly after removal from the chemostat. Samples were concentrated 5-fold in a speed-vac-concentrator prior to analysis. Before sampling, cultures were run for at least 5 vol changes at a set dilution rate.

4. RESULTS

4.1. Growth characteristics of Klebsiella *sp. strain K312,* Klebsiella terrigena *and* Citrobacter freundii

The nitrate dissimilating enterobacteria were grown in electron acceptor limited continuous culture (5 mM KNO_3) with glucose (40 mM) as carbon and energy source. Up to a dilution rate of $D = 0.12\ h^{-1}$ nitrate was reduced to ammonia. At a dilution rate of $D = 0.12$ to $0.16\ h^{-1}$, depending on the organism, nitrite accumulated and as a result of product inhibition, biomass concentration decreased. Concomitantly, the specific nitrite reductase activity decreased from 150 to 20 nmol $\times\ min^{-1} \times (mg\ protein)^{-1}$ whereas the nitrate reductase activity remained constant at 800 nmol $\times$ $min^{-1} \times (mg\ protein)^{-1}$ (average values of the 3 strains). Similar results were obtained with *Klebsiella* K312 and *Klebsiella terrigena* growing on glycerol (160 mM), and also with *Citrobacter freundii* growing on L-lactate (20 mM) as carbon sources.

For determination of some stoichiometric parameters, cultures were grown with glucose (20 mM), glycerol (80 mM) or L-lactate (60 mM) at a dilution rate of $D = 0.1\ h^{-1}$ (Table 1). The concentration of the electron acceptor was gradually increased (2.5–40 mM KNO_3). Growing on glucose, the 3 strains reduced nitrate to ammonia up to a C/N-ratio of 37.5 (g carbon/g nitrogen) without accumulation of nitrate and nitrate. If *Klebsiella* K312 was grown with glycerol as substrate, 75% of the nitrate added was recovered as ammonia. At a C/N-ratio lower than 6.8 (g carbon/g nitrogen), nitrite and, concomitantly, nitrate were detected in the culture fluid. The specific nitrate consumption rate was also significantly higher than in glucose culture due to the limited ability of *Klebsiella* K312 to ferment glycerol. For all strains tested, the ratio of the fermentation products, acetate and ethanol, was strongly affected by the C/N-ratio in glucose or glycerol cultures. An increase of the nitrate concentration resulted in a decrease of the ethanol/acetate ratio. The lowest C/N-ratio, 5.1 (g carbon/g nitrogen), was found in cultures of *Citrobacter freundii* growing with L-lactate, a non fermentable carbon and energy source. Only acetate was detected in the culture fluid.

4.2. Growth characteristics of P. stutzeri

The apparent half saturation constants for nitrate of *P. stutzeri*, obtained in electron acceptor-limited continuous culture, were 10 μM with glucose and 3 μM with L-lactate as carbon and energy source, respectively. Small amounts of nitrite (10–20 μM) were detected only at growth rates near μ_{max}.

Chemostat cultures were grown with glucose (20 mM) or L-lactate (20 mM) at a dilution rate of $D = 0.1\ h^{-1}$ at various nitrate concentrations (5–80 mM). Nitrate was completely denitrified to gaseous products up to C/N-ratios (g carbon/g nitrogen) of 1.71 and 2.0 with glucose and lactate, respectively. The results are shown in Table 1.

Table 1

Characteristics of *Pseudomonas stutzeri, Klebsiella terrigena, Klebsiella* sp. strain K312 and *Citrobacter freundii*

Strain	Substrate	μ_{max} (h^{-1})	Y (g dry weight/mol substrate)	q (mmol nitrate/g dry weight/hour)
P. stutzeri	Glucose	0.29	37.7	7.00
P. stutzeri	Lactate	0.31	14.6	7.10
K. terrigena	Glucose	0.56	16.5	0.66
K. 312	Glucose	0.79	23.8	0.81
K. 312	Glycerol	0.41	15.2	2.47
C. freundii	Glucose	0.74	18.9	1.32
C. freundii	Lactate	0.19	7.3	6.80

The growth experiments were performed in nitrate limited continuous cultures at a dilution rate of $D = 0.1\ h^{-1}$, pH 7.4 and 30 °C. The concentration of glucose and L-lactate were 20 mM, the concentration of the electron acceptor nitrate was 5 mM. In the case of *Klebsiella* K312 growing on glycerol (80 mM) the medium contained 30 mM nitrate.

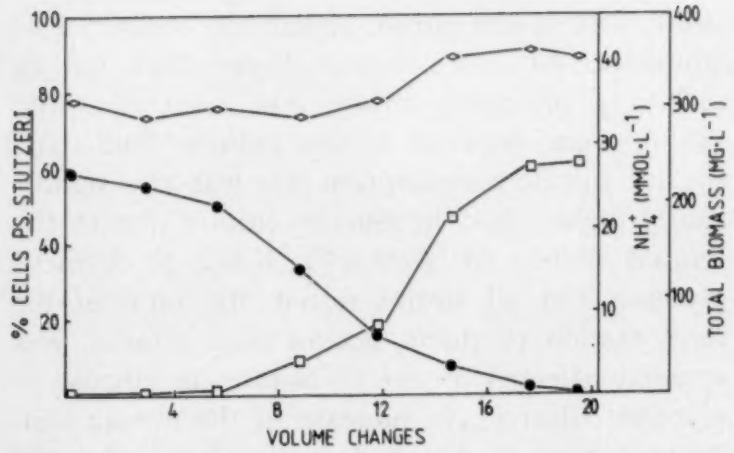

Fig. 1. Competition for nitrate between *Citrobacter freundii* and *Pseudomonas stutzeri* in an anaerobic electron acceptor-limited chemostat at a dilution rate of $D = 0.125\ h^{-1}$, pH 7.4 and 30 °C. The medium fed into the chemostat contained 60 mM L-lactate and 30 mM nitrate. ◇ = total biomass; ● = percentage of cells *Pseudomonas stutzeri*; □ = ammonia.

Neither alcohols nor fatty acids were detected in the culture fluid, therefore it is assumed that the carbon source was completely reduced to CO_2. Analysis of the composition of the gaseous products of denitrification was not made, since, according to [24], *P. stutzeri* reduces nearly all the nitrate respired to molecular nitrogen.

4.3. Competition for nitrate between C. freundii *and* P. stutzeri

At the beginning of each competition experiment, *C. freundii* and *P. stutzeri* were grown separately in continuous cultures as described in MATERIALS AND METHODS. The ammonia concentration, produced by *C. freundii*, never exceeded 3 mM. After steady state had been attained, both cultures were mixed, and the medium reservoir was simultaneously changed and the dilution rate adjusted.

C. freundii outcompeted the pseudomonad for nitrate with L-lactate as carbon and energy source at dilution rates of $D = 0.05\ h^{-1}$, $D = 0.1\ h^{-1}$, $D = 0.125\ h^{-1}$ and $D = 0.14\ h^{-1}$. Fig. 1 shows data of the experiment run at a dilution rate of $D = 0.125\ h^{-1}$. During the course of the competition, nitrate was not detectable. Under steady state conditions, 90 to 95% of the nitrate added was recovered as ammonia. At the end of each experiment, relative cell numbers (steady state condition) of *Pseudomonas* were 0.4 to 0.5% of the total counts.

Fig. 2 shows that competition for both nitrate and L-lactate at a dilution rate of $D = 0.05\ h^{-1}$ resulted in the coexistence of both species. Neither nitrite or lactate were detected in the culture fluid, with steady state concentrations of ammonia and acetate being 14.1 and 13.0 mM, respectively. The coexistence may be explained by the observation that pure cultures of *C. freundii* convert one mol L-lactate to a nearly equimolar amount of acetate with the concomitant reduction of 0.5 mol nitrate to 0.5 mol ammonia. Since the denitrifier was able to use acetate as carbon source and electron donor, *P. stutzeri* must have used a part of the acetate produced by *Citrobacter* for the reduction of the residual nitrate. An increase of the dilution rate up to $D = 0.1\ h^{-1}$ resulted in washout of *C. freundii* (data not shown).

Further competition experiments were undertaken in nitrate limited continuous cultures with glucose plus L-lactate, at varying concentrations, as carbon sources. Table 2 summarizes the results of such experiments (steady state conditions). Experiments were started with equal numbers of *Pseudomonas* and *Citrobacter*. Nitrate, nitrite and glucose were not detected during the course of the competition experiments. At a low dilution rate ($D = 0.05\ h^{-1}$), *C. freundii* appeared to be the

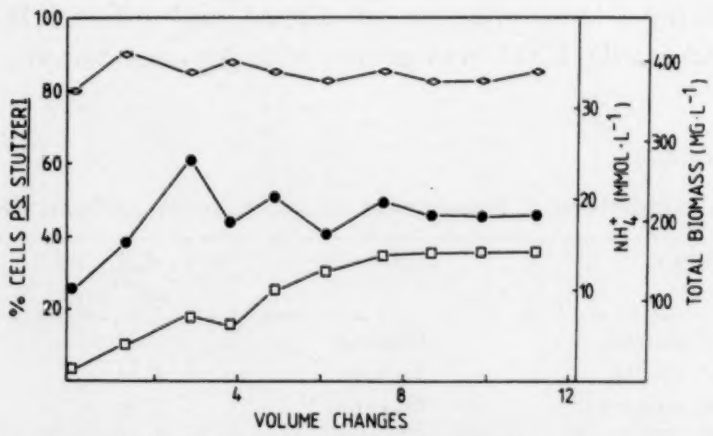

Fig. 2. Competition for nitrate and L-lactate between *Citrobacter freundii* and *Pseudomonas stutzeri* in an anaerobic electron acceptor-limited chemostat at a dilution rate of $D = 0.05\ h^{-1}$, pH 7.4 and 30 °C. The medium fed into the chemostat contained 30 mM L-lactate and 30 mM nitrate. ◇ = total biomass; ● = percentage of cells *Pseudomonas stutzeri*; □ = ammonia.

Table 2

Competition for nitrate between *Citrobacter freundii* and *Pseudomonas stutzeri* in an anaerobic electron acceptor-limited chemostat at various dilution rates, pH 7.4 and 30 °C

Additional glucose (mM)	Dilution rate (h^{-1})	Percentage of cells *Ps. stutzeri*	NH_4^+ (mM)	Total biomass (g/l)
5	0.06	4.0	23.0	0.48
10	0.06	0.5	24.0	0.54
20	0.06	0.4	19.0	0.79
5	0.12	55.0	0.1	0.42
10	0.12	8.9	4.1	0.59
20	0.12	0.1	22.0	0.89
20	0.28	25.0	0.3	1.03

The medium fed into the chemostat contained 30 mM nitrate, 30 mM L-lactate and different amounts of glucose as additional energy and carbon source.

best competitor of both strains. Using 5 mM glucose as additional carbon source, cells of *Pseudomonas* were washed out to a level of 4% of the total population. At a glucose concentration of 10 mM the pseudomonad was completely washed out of the chemostat. In cultures with 5 and 10 mM glucose as additional energy and carbon source ($D = 0.12\ h^{-1}$) both species coexisted, due to the ability of *Citrobacter* to ferment glucose. The major part of the nitrate, depending on the composition of the population in the mixed culture, was denitrified by the pseudomonas. An increase of the concentration of glucose up to 20 mM resulted in the washout of *P. stutzeri*. At a dilution rate of $D = 0.28\ h^{-1}$ near the maximum growth rate of *P. stutzeri*, a stable co-culture of both species was obtained after 40 vol. changes using 20 mM glucose as additional carbon source. Interestingly, all the nitrate was denitrified, despite the fact that the pseudomonad formed only 25% of the population.

5. DISCUSSION

For *P. stutzeri*, the apparent half saturation constant (K_s) of nitrate was 10 and 3 μM, respectively, growing in cultures on glucose and lactate as carbon and energy sources. A similar value, 3.2 μM, has been found in continuous culture for *Thiobacillus denitrificans* [23]. Due to the relatively high limits of nitrate detection (25 μM) and due to the inhibition of nitrate ammonifying bacteria by nitrite observed at dilution rates $D \geq 0.12\ h^{-1}$ it was impossible to determine the K_s-value for nitrate in continuous cultures of these bacteria. Obviously, the enterobacteria exhibit a greater nitrate than nitrite reduction rate. These in vivo observations are supported by the results of the in vitro nitrate and nitrite reductase assays. Of the 3 strains studied, *C. freundii* was chosen for competition experiments due to its ability to use L-lactate as electron donor for the dissimilatory nitrate reduction to ammonia. In mixed cultures of *C. freundii* and *P. stutzeri*, the nitrite produced was instantly used by the pseudomonad and its concentration never attained the inhibitory level. Thus, the inhibitory effect of nitrite, observed in pure cultures of the nitrate ammonifying bacteria, was eliminated in mixed culture.

Koike and Hattori [9] demonstrated that the organic content of coastal sediments influenced the products of nitrate dissimilation. On the basis of these observations, Tiedje et al. [14] suggested, that the balance between denitrification and nitrate dissimilation to ammonia is a function of the ratio of electron donor to electron acceptor. Our data are in accordance with this hypothesis, since in pure cultures of the enterobacteria and of *P. stutzeri* the C/N-ratio required for denitrification was shown to be much lower than that needed for nitrate ammonification. A similar explanation can be given for the outcome of the competition experiments. When competing for nitrate with the pseudomonad in cultures with sufficiently high concentration of the electron donor (L-lactate), *C.*

freundii was favoured at dilution rates up to D = 0.14 h^{-1}. This result also confirms that *C. freundii*, growing on L-lactate as sole electron donor, had a lower half saturation constant for nitrate than the pseudomonad. By simultaneously limiting the nitrate and lactate supply, a coexistence of nitrate ammonifying and denitrifying organisms resulted, due to the ability of the pseudomonad to use acetate as electron donor. When using L-lactate and glucose as carbon sources, the outcome of the competition depended on the C/N-ratio, and also on the dilution rate. This result may be explained by the ability of *Citrobacter* to ferment glucose and by the far greater nitrate consumption rate of *P. stutzeri* (Table 1), especially with glucose as carbon source and electron donor.

C. freundii is able to metabolize glucose and other fermentable substrates during competition for nitrate. This ability, shared with the other enterobacteria studied (data not shown), may give the nitrate ammonifying organisms an advantage in anaerobic sediments and soil, where fermentable substrates are available. By using these substrates the enterobacteria may increase their biomass, a process resulting in the consumption of a larger fraction of nitrate. This principle of microbial competitiveness has been discussed by Gottschal [25].

Finally, it should be stressed, that care must be taken to extrapolate the results of these laboratory experiments to field conditions. Our studies are models for the field situation, where the populations of microorganisms do not experience the balanced steady state conditions of the chemostat.

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Denitrification and nitrate-reducing bacterial populations in abandoned and reclaimed minesoils *

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1. SUMMARY

Microbial populations, nitrogen mineralization potentials, and denitrification enzyme activities were examined in two abandoned carbolithic minesoils. Numbers and activities of bacteria and fungi were lower in nonamended than in lime and/or fly ash amended sites. Rates of aerobic NO_3^- production (3 to 38 μg-N kg^{-1} h^{-1}) and anaerobic NO_3^- reduction to N_2O (5 to 68 μg-N kg^{-1} h^{-1}) were measured. Organisms capable of N_2O production under anaerobic soil conditions were present in low numbers, and their activity was restricted in part by low soil pH. Nondenitrifying nitrate-reducing bacteria were more diverse and in greater numbers than respiratory denitrifiers and may have been responsible for N_2O production in assays measuring denitrification enzyme activity.

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2. INTRODUCTION

Regeneration and cycling of soil nutrients by microbial activity is important in reestablishing productive soils after surface mining for coal [1,2]. Schafer et al. [3] have suggested the use of microbial activity measurements as indices for minesoil genesis. The ratio of microbial biomass carbon to soil organic carbon in a chronosequence of minesoils has been used recently to demonstrate that (more) than 50 years may be necessary before steady state conditions are reattained [4].

Despite the perceived importance of microbial activities to minesoil fertility and genesis, relatively few studies have examined microbial characteristics of minesoils. Early work by Wilson [5] demonstrated that the number, diversity, and respiration rate of microorganisms in pyritic mine spoils were lower than those in adjacent native soils. Lawrey [6] and Carrel et al. [7] observed slower decomposition of a variety of plant litters in minesoils compared with undisturbed sites. More recently, Stroo and Jencks [8,9] found that application of lime and fertilizers stimulated soil respiration and organic matter decomposition in acidic minesoils. The stimulatory effect of these additions appeared to be transitory, however, and

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soil fertility eventually was depleted in amended sites compared with unfertilized minesoils of the same age.

Available N limits both revegetation and organic matter decomposition, yet the production and fate of inorganic N in minesoils has rarely been investigated. Carbolithic materials may contain as much as 1.6% total N [10], but the amount potentially available to microorganisms and plants is much less. Black pyritiferous shales may release soluble nitrogen during active microbial oxidation of reduced sulfur compounds [11]. Nitrification was measurable in shales and minespoils, and the rates of net ammonification and nitrification were greater in vegetated compared with nonvegetated sites [12–14].

The potential for losses of nitrified N from minesoils by denitrification is largely unknown. Denitrifying bacteria reduce NO_3^- and NO_2^- to the gaseous products N_2O and N_2, resulting in average annual losses of as much as 20–30% of plant available N [15]. Nondenitrifying heterotrophic bacteria also can produce N_2O as a result of NO_2^- reduction. However, the magnitude of gaseous N production by these bacteria currently is unknown in any soil [16,17]. The objective of the current work was to evaluate the potential for gaseous N production as a result of NO_3^- reduction in abandoned and reclaimed minesoils. We enumerated and identified bacteria from each site and characterized their ability to reduce NO_3^-. The effect of pH on gaseous N production by selected nitrate-reducing bacterial isolates was also examined.

3. MATERIALS AND METHODS

3.1. Site description

Soils were collected in 1985 and 1986 from the surface 0–20 cm of two abandoned minesites in Mineral County, WV (Bald Knob site) and Monongalia County, WV (Osage site). Minesoils were derived from acidic carbolithic shales and classified as loamy-skeletal, mixed, acid, mesic Typic Udorthents. The Bald Knob site was actively mined from 1965 to 1970 and is located 10 km northeast of Mt. Storm, WV (39°31′N, 79°12′W) at an elevation of 792 m. Two locations were sampled at this site: a nonamended area, and a research plot amended in September, 1979 with lime (10.2 Mg ha^{-1}), P (0.043 Mg ha^{-1}), and NH_4NO_3 (0.22 Mg Ha^{-1}) described by Vandevender and Sencindiver [18]. The Osage site is a coal mine refuse dump located 10 km north of Morgantown, WV (39°40′N, 80°03′W) at an elevation of 290 m. Three locations were sampled: a nonamended site, a site amended with pH 12 fly ash, and an area amended with both fly ash and lime. Fly ash (336 Mg ha^{-1}) was applied in 1971 as described by Capp [19].

Plant cover on nonamended soils at Bald Knob and Osage was less than 1%, consisting primarily of broomsedge (*Andropogon virginicus* L.). Plant cover on amended soils at both sites was estimated visually to be 80%, consisting primarily of a mixture of birdsfoot trefoil (*Lotus corniculatus* L.) and tall fescue (*Festuca arundinacea* Schreb.). Only unvegetated areas were sampled on nonamended sites, whereas vegetated areas were sampled from amended sites.

Four 2-kg subsamples were collected from each site. At the Bald Knob sites, single subsamples from each of four 9 m^2 treatment plots were collected and pooled. The four subsamples from the Osage sites were randomly collected from a 100 m^2 area. All samples were passed through a 4.75 mm sieve and placed in airtight Ziploc plastic bags and stored field moist at 4°C prior to microbiological analyses. Chemical characteristics of the minesoils (Table 1) were determined by standard procedures at the Cornell University Nutrient Analysis Laboratory (Cornell University, Ithaca, NY).

3.2. Microbiological characteristics of minesoils

Populations of fungi, yeasts, total bacteria and aerobic spore-forming bacteria were enumerated in each soil by spread plating onto microbiological media from Difco (Difco Laboratories, Detroit, MI). Fungi and yeasts were cultured on peptone-glucose acid agar and Czapek-Dox-sodium proprionate agar, respectively [20]; total bacteria on trypticase soy agar amended with 50 $\mu g\ ml^{-1}$ cyclohexamide; and aerobic spore-forming bacteria on glucose-nutrient agar after heat-shock-

ing diluted soil samples at 80 °C for 10 min [20]. Samples were replicated in triplicate for all spread plate procedures and incubated at 30 °C for 48 h prior to counting.

Nitrate-reducing bacteria were enumerated by a 5 tube MPN procedure using 10 ml of nutrient broth and 5 mM KNO_3 in Hungate tubes [21]. Tubes were incubated at 30 °C for 14 days. Two 1-ml broth samples from each MPN tube were added to spot plates and analyzed for NO_3^- and NO_2^-. A blue color with addition of diphenylamine was scored positive for NO_3^- plus NO_2^-. A red color after addition of sulfanilamide and *N*-(1-naphthyl)-ethylenediamine was scored positive for NO_2^-. If a tube was negative for NO_2^-, zinc dust was added and a red color change was scored positive for NO_3^-.

3.3. Net nitrogen mineralization potentials

The nitrogen mineralization potential assay is an index of the nitrogen supplying power of a soil measuring the net amount of nitrogen mineralized to NH_4^+ and nitrified to NO_3^- during a laboratory incubation [22,23]. Eight 10-g samples of each soil were placed into individual 250 ml Erlenmeyer flasks and covered with plastic wrap. A pencil-sized hole was punched into each cover to allow free gas exchange with the atmosphere. Soils were wetted to their gravitational water holding capacity. Flasks were incubated at 25 °C, weighed daily, and water added to maintain soil gravimetric moisture content. At 0 and 30 days duplicate flasks were analyzed for NH_4^+ and NO_3^- production. NH_4^+ was extracted with 1 N KCl and analyzed on a Technicon autoanalyzer (Technicon Instruments Corp., Tarrytown, NY). Nitrate was extracted with 0.01 N KCl and analyzed on a Dionex ion chromatograph (Dionex Corp., Sunnyvale, CA). Rates were calculated from the difference between the mean concentrations of extractable NH_4^+ and NO_3^- at day 0, and at day 30.

3.4. Denitrification enzyme activity

The relative quantity of functioning denitrifying enzymes in each soil was determined using a modification of the procedures described by Smith and Tiedje [24] and Tiedje [21]. A solution (25 ml) containing 5 mM glucose, 5 mM NO_3^-, and 1 g chloramphenicol per liter was added to replicate 158 ml serum bottles containing 20 g of field moist soil. Sterile control soils were prepared by autoclaving field moist soil samples in the serum bottles (120 °C, 30 min) prior to adding the solution. Some sterile control bottles received a solution containing 5 mM NO_2^- in place of NO_3^- after autoclaving. In some experiments, pH of the soil slurry was adjusted with either 1 N HCl or 1 N NaOH. The bottles were sealed with black rubber stoppers, evacuated, and then flushed 3 times with argon. Acetylene was injected into the bottles (final concentration, 20% v/v) and N_2O production was monitored over a 4- to 8-h period. All samples were incubated at room temperature (24°–26 °C). The rate of N_2O production was measured by periodically injecting 0.2 ml of headspace gas into a gas chromatograph. Samples containing < 30 ppm (v/v) N_2O were measured with a Varian 3700 gas chromatograph (Varian Associates, Walnut Creek, CA) equipped with a ^{63}Ni electron capture detector. Concentrations $>$ 30 ppm were determined using a Carle series 100 gas chromatograph (Hach Company, Loveland, CO) equipped with a thermal conductivity detector. Quantities of N_2O in solution were calculated using published values of the Bunsen absorption coefficient [21].

3.5. Bacterial isolations

A 20-g sample from each soil was placed in a sterile 250-ml Erlenmeyer flask, sealed with a rubber stopper, and made anaerobic by replacing headspace gas with argon. Following a 3-day anaerobic incubation, 180 ml of sterile 0.1% peptone buffer and a drop of Tween 80 were added and the flasks placed on a rotary shaker at 120 rpm for 5 min. Samples were diluted in sterile 0.1% peptone buffer and 0.1 ml aliquots were spread plated on replicate ($n = 5$) nutrient agar (BBL Microbiology Systems, Cockeysville, MD) plates supplemented with 5 mM KNO_3 and 50 μg ml^{-1} cyclohexamide (Sigma Chemical Co., St. Louis, MO). Plates were anaerobically incubated in BBL GasPak jars for 4 days at room temperature. Isolated colonies were selected from plates containing 10–100 colonies, until the desired 70–80 isolates were obtained. All isolates were

maintained on nutrient agar supplemented with 5 mM KNO_3 in screw cap tubes at 4°C.

Isolates were screened for ability to reduce NO^{-3} in Bacto-tryptic nitrate medium (Difco Laboratories, Detroit, MI). Presence or absence of NO_2^- or NO_3^- was determined using procedures described in section 3.2. Nitrate-reducing isolates were evaluated for colonial pigmentation, Gram-stain, morphology, size, motility, spore formation, oxidase and catalase activity, oxygen requirement, citrate utilization, mixed acid fermentation, and production of acetylmethylcarbinol, indole, and H_2S.

Isolates were divided into 5 characteristic groupings. Group I isolates were Gram positive, facultatively anaerobic, filamentous actinomycetes in the genus *Streptomyces*. Group II isolates were Gram-positive, endospore-forming, respiratory-aerobic, motile, rod shaped bacteria in the genus *Bacillus*. Group III isolates were facultatively anaerobic *Bacillus* spp. Gram-negative bacterial isolates in Group IV and V were characterized further by API 20E identification strips (Analytab Products, Plainview, NY) and examined for the production of fluorescent pigments after 48-h growth at 27°C on BBL Tech agar (BBL Microbiology Systems, Cockeysville, MD). Group IV isolates were a heterogeneous group of Gram-negative, facultatively anaerobic, oxidase negative, catalase positive, nonpigmented, motile rods in the Enterobacteriaceae. Representative isolates in Group IV most closely resembled the genera *Enterobacter* and *Citrobacter,* however, not all Group IV isolates examined were identifiable by the API 20E identification system. Group V isolates appeared to be homogenous, consisting of Gram-negative, respiratory-aerobic, oxidase positive, catalase positive, nonpigmented, nonfluorescent pigment producing, motile rods resembling *Alcaligenes* species.

3.6. Nitrate-reducing activity of bacterial isolates

The products of nitrate reduction in the presence or absence of acetylene (10% v/v) were quantified for 15 isolates representative of the 5 groups described in section 3.5. Autoclaved rubber-stoppered tubes, containing 9.3 ml of nutrient broth (BBL Microbiology Systems, Cockeysville, MD) and 5 mM KNO_3 adjusted to pH 6.5 with 1 N HCl, were inoculated with 0.1 ml of an 18–24 h broth culture of each isolate. Uninoculated tubes were included as a control. Tubes were made anaerobic by replacing the headspace gas with argon. After 10 days, broth samples of each isolate were diluted 1 : 17 with Milli-Q Type I reagent grade water (Millipore Corp., Bedford, MA). NO_3^- and NO_2^- were determined colorometrically with a Perkin-Elmer 55B spectrophotometer (The Perkin-Elmer Corp., Norwalk, CN) using the Szechrome NAS procedure (Polysciences, Inc., Warrington, PA), and a modified Griess-Ilsovay method [25], respectively. Headspace gas samples were analyzed for N_2O as described previously in section 3.4.

Patterns of NO_3^- reduction among the isolates were grouped by the criteria of Smith and Zimmerman [16]. Isolates which produced NO_2^- as the major product of NO_3^- reduction were called NO_2^- accumulators. Isolates which stoichiometrically reduced NO_3^--N to N_2O in the presence of acetylene were classified as respiratory denitrifiers. Isolates for which less than 60% of the NO_3^- was recovered as NO_2^- and N_2O were assumed to produce NH_4^+ as a result of NO_2^- reduction.

Twelve isolates were tested to determine the effect of pH on growth and nitrate-reducing activity. The growth medium described above was adjusted to pH 6.5, 5.5, 4.5, or 3.5 ($\pm$0.05) with 1 N HCl. Bacterial growth after 10 days was estimated spectrophotometrically at 520 nm with a Spectronic 20 (Bausch and Lomb, Rochester, NY). Headspace N_2O and residual broth NO_3^- and NO_2^- were quantified as described previously in sections 3.4 and 3.6, respectively.

4. RESULTS AND DISCUSSION

4.1. Chemical and microbiological characteristic of minesoils

The coal mining overburden at Bald Knob and Osage contained oxidizable pyrite which contributed to soil acidity. Twenty years after mining, exchangeable acidity was higher and pH was significantly lower in unamended than in adjacent amended minesoils (Table 1). The number of

Table 1

Physical and chemical characteristics of Bald Knob and Osage minesoils

Site [a]	% Moisture [b]	pH	Exch. acid. (cmol + kg^{-1})	Available (mg kg^{-1})									Total N (%)	Organic carbon (%) [c]
				Fe	Al	Mn	Mg	P	K	Ca	NO_3^--N	NH_3-N		
BK NA	10.3	3.0	23	103	263	1	13	<1	12	103	9	5	0.2	7
BK AM	11.5	6.8	ND [d]	12	130	2	25	<1	59	5533	15	4	0.2	10
OS NA	9.8	2.7	17	388	57	3	18	7	<6	763	3	2	0.9	30
OS FL	11.9	6.4	ND	22	18	19	79	10	60	3462	21	7	0.9	30
OS FA	26.5	5.2	11	45	77	3	91	11	69	905	24	6	0.7	39

[a] BK NA, Bald Knob nonamended; BK AM, Bald Knob amended; OS NA, Osage nonamended; OS FA, Osage amended (fly ash); OS FL, Osage amended (fly ash and lime). [b] Gravimetric moisture content determined in our lab immediately following sampling. [c] The high % organic carbon reflects the presence of carbolithic shales in these minesoils. [d] ND, nondetectable.

bacteria and fungal propagules were significantly lower and the percentage of the bacterial population represented by aerobic spore-forming bacteria generally was higher, in the unvegetated compared with the vegetated sites (Table 2). These data are consistent with previous studies that found lower numbers and activities of microorganisms in acidic minesoils than in adjacent reclaimed soils [5,7–9,26].

Nitrate-reducing bacteria, enumerated by an MPN procedure, represented less than 3% of the total heterotrophic plate count in each minesoil (Table 2), and numbers of nitrate-reducing bacteria always were significantly lower in nonvegetated sites. The ability to reduce NO_3^- normally is widely distributed among genera of soil bacteria, and the low percentage of the total heterotrophic population represented by nitrate reducers may reflect limited diversity of bacteria in minesoils. The MPN procedure enumerated both denitrifying and nitrate-respiring bacteria [21]. Denitrifying bacteria normally comprise up to 5% of enumerable soil heterotrophs [27], and nitrate-respiring bacteria often outnumber denitrifiers 4:1 in undisturbed soils [16,28]. Nitrate-reducing populations have been examined in low pH soils [29], but are not well characterized in minesoils. Numbers of denitrifying bacteria in acidic minesoils reported by Wilson and Stewart [12] were significantly higher than those reported in the present study, but non-specificity of the MPN procedure may have overestimated counts of denitrifying bacteria [21,30].

Table 2

Microbiological characteristics of Bald Knob and Osage minesoils

Site [a]	Fungi (propagules g^{-1}) [b,c]	Aerobic spore-forming bacteria (CFU g^{-1}) [c]	Total bacteria (CFU g^{-1}) [c]	Nitrate reducer MPN (MPN Index g^{-1}) [d]
BK NA	1000	<10	130	3
BK AM	27000	240000	1400000	180
OS NA	40	470	47	6
OS FL	4200	450000	560000	3600
OS FA	41000	1100000	2200000	980

[a] Site designations are described in Table 1. [b] Yeast <10 CFU g^{-1} soil. [c] Mean values of 3 replicates. [d] 5 tube MPN Index.

4.2. Nitrogen mineralization potential

Availability of inorganic nitrogen is a major factor limiting plant establishment and microbial activity in minesoils [31]. Carbolithic shales contain up to 1.6% total nitrogen [10], but only a small portion of this nitrogen can be mineralized to plant-available forms [11,13]. Soils from the Osage and Bald Knob sites averaged 0.9 and 0.2% total nitrogen, respectively, and extractable NO_3^--N and NH_4^+-N generally were higher in amended than unamended soils (Table 1). Although net nitrification was measurable in all soils, nitrification rates were lower in the unamended soils. This resulted in a net accumulation of NH_4^+ and less

Table 3

N mineralization and denitrification activities of Bald Knob and Osage minesoils

Site [a]	N mineralization potential (μg-N kg^{-1} h^{-1}) [b]		Denitrification enzyme activity (μg-N kg^{-1} h^{-1}) [c]
	NH_4^+	NO_3^-	
BK NA	20.8	3.3	4.6 (0.4)
BK AM	−12.5	37.5	10.7 (0.9)
OS NA	33.3	8.3	11.1 (1.5)
OS FA	−8.3	20.8	67.6 (9.0)

[a] Site designations are described in Table 1. [b] Rates determined from the mean ($n = 2$) differences between the ion pool size at day 0 and day 30. [c] Mean values of 8 replicates for Bald Knob sites and 13 replicates for Osage sites; numbers in parentheses are standard errors.

accumulation of NO_3^- (Table 3). Low nitrification rates appear to be common in acidic minespoils [5,32,33], presumably due to inhibition of chemoautotrophic nitrifying bacteria by low soil pH [34]. Recent studies demonstrated heterotrophic fungi are potentially the important nitrifying microorganisms in low pH forest soils [35,36]. Whether the low rate of NO_3^- accumulation we observed in the unamended acidic minesoils was attributable to autotrophic or heterotrophic nitrification remains to be determined.

4.3. Denitrification enzyme activity

NO_3^- formed by nitrification can become unavailable to plant uptake by leaching, immobilization, or denitrification. Nitrogen immobilization is favored in carbolithic minesoils due to the high C : N ratios of these materials (Table 1) [14], and leaching of NO_3^- from minesoils has been documented [37]. In contrast, the potential for nitrogen loss from minesoils due to denitrification has not been assessed. Short term (4 h) enzyme activities demonstrated denitrifying potential in minesoils from Bald Knob and Osage (Table 3). The rate of denitrification ranged from 3 to 20 μg N_2O-N kg^{-1} h^{-1} in nonamended soils, as compared to rates ranging from 7 to 140 μg N_2O-N kg^{-1} h^{-1} in vegetated minesoils. In acid to neutral agricultural and forest soils denitrification activities are higher, ranging from 40 to 4000 μg N_2O-N kg^{-1} h^{-1} [27]. Since sterile minesoils incubated in the presence of either 5 mM NO_3^- or NO_2^- did not produce measurable N_2O, low activities could not be attributed to chemodenitrification of NO_2^- [38].

Carbon, NO_3^-, and denitrifying bacteria were homogenized in the anaerobic slurry, which estimates a maximum denitrification rate for the sample, and is indicative of the relative number of active denitrifying bacteria [21,24]. The low number of nitrate-reducing bacteria (Table 2) and low denitrification activities (Table 3) indicate that denitrifying bacteria are restricted in acid and reclaimed minesoils. Actual in situ denitrification N-losses were not determined in this study, but they usually are more than 100 × lower than those measured by the denitrification enzyme activity assay [39].

The activity of denitrifying bacteria in the unamended Osage minesoil appeared to be limited by soil acidity, since incubations at higher pH increased denitrification rates (Fig. 1). In contrast, denitrification enzyme activity in the fly ash amended soil was highest near the in situ pH and declined at higher soil pH. The optimum pH for soil denitrification and pure cultures of denitrifying bacteria generally falls between pH 6 to 8 [40]. However, denitrification potential also has been

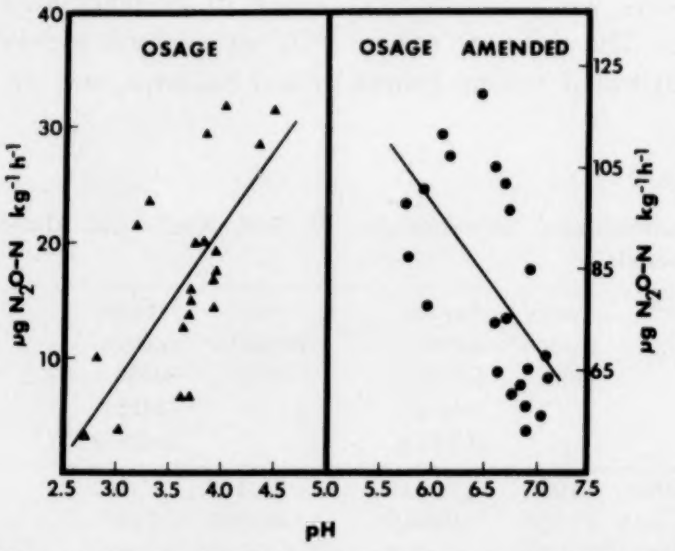

Fig. 1. Response of denitrification enzyme activity to pH. Denitrification enzyme activity was linearly correlated with pH in nonamended (Osage; $r = 0.69$, $p < 0.01$) and fly ash amended (Osage Amended; $r = -0.66$, $P < 0.01$) Osage minesoils. Note scale differences for denitrification rates of the two soils.

Table 4

Nitrate reduction by bacterial isolates

Site [a]	Number of isolates	NO_3^- reduced to:		
		NO_2^-	Beyond NO_2^- [b]	Not reduced
BK NA	70	35	1	34
BK AM	72	16	1	55
OS NA	72	19	10	43
OS FL	75	34	11	30
OS FA	75	38	16	21
TOTAL	364	142	39	183

[a] Site designations are described in Table 1. [b] N_2, N_2O, or NH_4^+.

measured in acid soils [41,42]. Unlike the work reported by Parkin et al. [42], the results of our study do not indicate the presence of a population of denitrifying bacteria exhibiting a low pH optimum as a result of adaptation to long term conditions of low soil pH. However, measurable denitrification activity in the unamended minesoils suggests the presence of denitrifying bacteria which are tolerant of low soil pH.

4.4. Characterization of nitrate-reducing bacterial isolates

Of the 364 bacterial isolates screened for NO_3^- reduction, 181 isolates were positive. Overall, 142 isolates accumulated NO_2^-, while the remaining isolates reduced NO_3^- to NH_4^+ or N_2 gas (Table 4). The majority (172) of the nitrate-reducing isolates could be categorized into 5 groups based on morphological and biochemical tests. Using the criteria of Smith and Zimmerman [16], isolates from Group I, II, and IV were classified as NO_2^- accumulators; Group III isolates were capable of fermentative dissimilatory NO_2^- reduction to NH_4^+ and Group V isolated were respiratory denitrifiers (Table 5).

Gram-positive *Bacillus* spp. (Group II and III) were the most common nitrate-reducing isolates obtained in amended and nonamended minesoils, followed by members of Group IV. The predominance of *Bacillus* spp. is consistent with the high percentage of heterotrophic spore-forming bacteria in all minesoils (Table 2). It was not determined if these isolates were representative of an active population of vegetative cells or reflected persistence of endospores. Group II isolates were nonfermentative, reduced less than 25% of the available NO_3^-, and did not produce measurable N_2O. In contrast, Group III isolates were facultative anaerobes which produced a mixture of NO_2^- and NH_4^+ and also converted as much as 25% of the available N to N_2O. Group IV isolates, like Group II isolates, were NO_2^- accumulators, but they produced measurable N_2O. Previous investigators have reported that isolates such as *Bacillus, Enterobacter,* and *Citrobacter* are the most numerous nitrate-reducing bacteria recoverable from

Table 5

Characterization of nitrate-reducing isolates

Nitrate-reducing activity	Group	Genus/Family	Total no. of nitrate-reducing isolates [a]	Nitrate reduction patterns (μg *N*): [b]							
				With acetylene				Without acetylene			
				NO_3^-	NO_2^-	N_2O	Total *N* recovered (%)	NO_3^-	NO_2^-	N_2O	Total *N* recovered (%)
Nitrite respiration	I	*Streptomyces* spp.	20 (0)	345	261	<1	93	360	273	<1	97
	II	*Bacillus* spp.	10 (3)	485	65	<1	84	484	4	<1	75
	III	*Bacillus* spp.	102 (41)	66	222	86	57	<1	244	66	48
	IV	Enterobacteriacae	26 (17)	109	451	51	94	38	507	46	91
Denitrification	V	*Alcaligenes* spp.	14 (0)	<1	5	528	85	<1	<1	<1	<1

[a] Number in parentheses is the number of total nitrate-reducing isolates obtained from unamended minesoils. [b] Nitrogen recovery for one individual isolate representative of the entire group; number of isolates tested for each group was: Group I, 2; Group II, 2; Group III, 4; Group IV, 3; Group V, 4.

Table 6

Nitrate reduction patterns of representative Group isolates at varying pH

Group [a]	pH [b]	Optical density (520 nm)	Recovered N (μg) as: [c]			Total *N* recovered (%)
			NO_3^-	NO_2^-	N_2O	
I	6.5	0.020	336	43	<1	105
	5.5	0.020	329	<1	<1	91
	4.5	0.050	367	<1	8	104
II	6.5	0.180	18	240	55	87
	5.5	0.050	337	5	<1	95
	4.5	0.070	370	<1	3	104
III	6.5	0.220	23	226	48	83
	5.5	0.180	27	216	15	72
	4.5	0.130	193	93	2	80
IV	6.5	0.440	35	232	48	88
	5.5	0.290	10	258	29	83
	4.5	0.170	22	243	34	83
V	6.5	0.700	<1	18	425	123
	5.5	0.840	<1	12	309	89
	4.5	0.200	331	34	2	102

[a] Groups are described in Table 5. [b] No isolate grew at pH 3.5. [c] Number of isolates tested for each group was: Group I, 2; Group II, 1; Group III, 2; Group IV, 3; Group V, 4; Data from only one isolate per group is presented.

neutral and acidic agricultural soils and can produce N_2O as a result of NO_2^- reduction [16,28,43–45]. Production of N_2O by these bacteria has been termed non-respiratory denitrification, but the significance of this process as a mechanism for gaseous N-loss from soils is currently unknown [17].

Group I and Group V isolates were obtained only from amended minesoils, primarily at Osage. Group I isolates accumulated NO_2^- as the major end product of NO_3^- reduction, generally reducing 50% of the available NO_3^- in the assay. In contrast to members of Groups III and IV, isolates in Group I produced little N_2O from NO_2^-. Although NO_3^- reduction is a common feature of Actinomycetes [46], the activity of environmental isolates rarely has been reported.

None of the isolates in Group V were fluorescent pseudomonads which previously have been reported as the numerically dominant denitrifiers in a variety of soils when isolated on nitrate agar [16,28,45]. Based on the lack of carbohydrate utilization these isolates were identified tentatively as *Alcaligenes* sp. In the study by Gamble et al. [28] all soils tested yielded at least 2 groups of denitrifiers and frequently many more. It would appear that in addition to numbers and activities, the diversity of denitrifying bacteria is more restricted in minesoils than in undisturbed soils.

No isolate from any group grew at pH 3.5 in NO_3^- broth. Isolates from all groups exhibited maximum growth and NO_3^- reduction at pH 6.5, but varied in their response to lower pH (Table 6). Groups I and II isolates grew poorly and did not reduce NO_3^- below pH 6.5. Group III isolates from all sites produced less NO_2^- and N_2O at pH 4.5, as was observed for a *Bacillus* sp. by Smith and Zimmerman [16]. Group IV isolates grew equally well, and produced similar amounts of NO_2^- and N_2O, regardless of incubation pH. Group V isolates stoichiometrically reduced NO_3^- to N gases at pH 5.5 and above. However, these isolates exhibited lower total growth and accumulated NO_2^- as the major end product of NO_3^- reduction at pH 4.5. Similar results were reported for an environmental isolate of the denitrifier *Pseudomonas fluorescens* when incubated at reduced culture pH [45].

4.5. Sources of N_2O production in minesoils

The absence of denitrifying isolates from nonamended sites, and the relatively small percentage of denitrifiers from amended sites contrasts with low but measurable denitrification enzyme activity present in all minesoils examined. Incubations with sterile controls indicated the observed denitrification activity was not due to chemical production of N_2O indicating a biological source of this gas in these soils. We cannot unequivocally conclude that nitrate-reducing isolates obtained from any site were responsible for denitrification activity of soil slurries. Rich laboratory media are selective for denitrifying bacteria which grow rapidly in the presence of excess carbon, and these organisms may not grow as denitrifiers in the natural environment [15]. The absence of denitrifying isolates from nonamended low pH sites is not conclusive since isolates were screened in a culture medium of neutral pH. In separate enrichment studies, acid tolerant denitrification activity has not persisted during laboratory subculture (A.J.S., unpublished data).

Some of the N_2O may have been produced in the anaerobic soil slurries by nondenitrifying nitrate-reducing isolates from Groups II, III, and IV. These organisms were obtained from all minesoils in greater numbers than respiratory denitrifiers, reduced as much as 25% of the NO_3^--N to N_2O, and produced N_2O at low pH. Smith and Zimmerman [16] observed immediate production of N_2O when similar bacteria were added to anaerobic slurries of autoclaved soils. N_2O produced by these organisms would be indistinguishable from that produced by denitrification, particularly in acid soils where N_2O is often the terminal denitrification product [40,42,47]. Selective inhibitors to partition N_2O production by the two processes are not currently available [48]. The rate of nitrate reduction and N_2O production by respiratory denitrifiers would normally be expected to greatly exceed that of nondenitrifying nitrate reducers in soil [17]. However, computer simulations based on published kinetic parameters suggest that if the population density of nondenitrifying nitrate reducers is large relative to denitrifiers, then the former can effectively compete for soil NO_3^- [27]. Data presented in the current study demonstrated that denitrifying populations are restricted, and suggest that nondenitrifiers may be the dominant bacteria capable of anaerobic NO_3^- utilization in minesoils.

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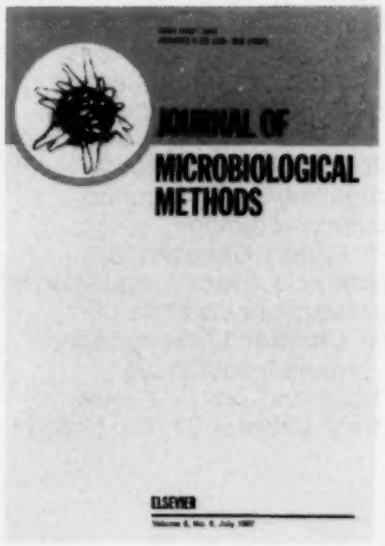

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